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Evaluation of the Photometric Assay Method to Quantitate the Bactericidal Action of Normal and Immune Rabbit Sera on SALMONELLA PULLORUM

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EVALUATION OF THE PHOTOMETRIC ASSAY METHOD TO QUANTITATE THE BACTERICIDAL ACTION OF NORMAL AND IMMUNE RABBIT SERA ON SALMONELLA PULLORUM

by

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I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in the Field of Microbiology.

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CHAPTER I

INTRODUCTION

Purpose

In previous studies in this laboratory, attempts had been made to detect Salmonella pullorum antibodies in serum and other body fluids by slide and tube agglutination test with unsatisfactory results, particularly when the antibody was in very low concentration. It was desired to find a test for the detection of the specific antibody which would be more sensitive than the commonly used agglutination test.

Muschel and Treffers (1956) published a photometric assay method for determining the bactericidal action of normal or immune serum using Salmonella typhosa. The study reported here was conducted for the purpose of determining whether the same method could be used for detecting and quantitating antibodies against Salmonella pullorum. It was hoped that the photometric assay technique would provide a very sensitive, reproducible, and practical method. Thus, their method was first adapted to the Salmonella pullorum system and then applied by determining the antibody content of the sera of several rabbits throughout an immunization program. Immunization curves were used to demonstrate the development of bactericidal antibody in response to the immunization. An evaluation of the sensitivity of the method includes a comparison of the bactericidal and agglutinating action of the sera of the rabbits.

Principle

When an animal comes into contact with an antigenic substance, by either natural or artificial means, the response usually includes the production of a specific antibody. The presence of the specific antibody can be demonstrated in vitro in several ways depending upon the physical nature of the antigen and antibody. Common reactions include agglutination, precipitation, complement-fixation, phagocytosis and the killing (bactericidal) action of certain gram negative bacilli. Most antibodies present in the serum of the animal will react with their specific antigen in vitro to produce a visible reaction. The bactericidal action of complement in the presence of immune serum, is one of the most sensitive methods for detecting the presence of specific antibodies. Thus, bactericidal antibodies, when present, can usually be detected in much smaller concentrations than can agglutinating and precipitating antibodies. Bactericidal antibodies are much more easily detected than phagocytic antibodies and they are thought to be of greater significance as a natural protective mechanism to the host animal than are complement-fixing antibodies.

The bactericidal antibody test is dependent on the presence of sufficient complement, magnesium ions, as well as the specific antibody, to produce lysis, or killing without lysis, of certain gram negative bacilli. Gram positive bacteria are resistant to this action.

This action will occur only in the presence of complement, a thermolabile and non-specific protein of mixed globulin composition which is present in all mammalian sera. The antibody reacts by combining firmly with the living bacterium (antigen) to form a sensitized antigen-antibody complex. The complement then combines non-specifically with this complex. If sufficient complement is present, the bacterium of the complex is lysed or killed. The antibody and complement have no independent combining affinity and the antigen and complement only a very weak combining affinity. It has thus been supposed that the antibody acts by sensitizing the bacterium to the action of complement, and that complement is the agent which causes the lysis or killing.

Several quantitative methods for measuring the bactericidal antibody content of normal and immune serum for gram negative bacilli have been introduced in recent years. All of these methods are based upon an assay of the number of bacteria which survive exposure to the killing effect of antibody plus complement. They include the method of Muschel and Treffers (1956), Nagington (1956), Landy et al. (1962), and Amano (1958). All of these methods have advantages and the method selected may be a matter of individual preference. For this study the turbidimetric assay method of Muschel and Treffers (1956) was selected for the following reasons:

1. It is a quantitative method for the detection of bactericidal antibody.
2. It is more reproducible than the plate count method.
3. It is a rapid method with results obtainable within 5 hours.
4. It has high sensitivity and reproducibility.
5. It uses an excess of complement and therefore is applicable for testing highly immune serum.

Immunized animals respond with much higher titers of bactericidal effect. Thus, the serum of an immunized animal may produce a 50% survival end point titer of 1:52,000 as opposed to a titer of 1:8 before immunization. The specific bactericidal component of normal and immune serum is a specific antibody which increases in thermostability as a result of immunization (Muschel and Treffers, 1956). It appears in the very young animal even before the appearance of other natural antibodies such as agglutinins and hemolysins. There is evidence of their presence in the sera of germfree animals (Muschel and Osawa, 1960, Michael et al., 1962).

In the photometric assay method a standardized minimal number of living bacteria are placed in contact with the serum containing the antibody and the guinea pig complement for a prescribed interval of time, termed the reaction time. After this period, an excess of nutrient broth is added which will dilute and inactivate all remaining complement, thereby stopping any further killing. All surviving

viable bacteria, will then grow and multiply in the broth upon incubation. The per cent of bacteria surviving the killing action is measured by determining the amount of turbidity developed during a definite incubation period, as the amount of turbidity, expressed in units of optical density, will be directly proportional to the number of viable bacteria present at the start of incubation. The results are then expressed as that amount of serum (antibody) which will kill 50% of the initially inoculated bacteria during the reaction time or result in 50% survival. The 50% survival end point can also be expressed in terms of serum titer. The serum titer is the reciprocal of the amount of serum producing a 50% survival end point and is that dilution at which 1 ml of serum would give the 50% survival end point. Thus, a 50% survival end point of 0. 25 ml of a 1:500 dilution can also be expressed as a serum titer of 2000.

Normal serum of most animals will exhibit a low titer of bactericidal effect in vitro. Mackie and Finkelstein (1932) found that although the sera of some animal species possessed far greater killing capacity for susceptible gram negative bacilli than did the sera of other animal species, that individual animals of the same species might vary considerably in the bactericidal action of their sera. Roantree (1960) found the rate of killing action of susceptible bacteria to be relatively constant for different animals of two animal species tested.

Selection of the Salmonella pullorum System

Salmonella pullorum was selected as the antigen for this study because, under natural conditions, it occurs rarely in the environment of man and experimental mammalian animals and is associated naturally only in fowl. Thus, a very low level of natural antibody would be expected to occur in man or any mammalian experimental animal. If infecting doses are ingested by man, it has a relatively low level of pathogenicity as compared to Salmonella typhosa and Shigella dysenteriae, thus risk of infection to laboratory personnel is at a minimum. At a later date, it is desired to use this organism in further study of bovine immunization as well as on human subjects.

The rabbit was chosen as the experimental animal to be immunized for convenience and because the bactericidal action of normal rabbit serum closely resembles that of normal human serum (Roantree and Pappas, 1960).

CHAPTER II

REVIEW OF LITERATURE

Early Developments (1894-1948)

Throughout the past sixty-nine years many studies have been reported regarding the bactericidal antibody and complement system for killing gram negative bacilli. The discovery of the extracellular bactericidal effect of immune serum referred to as the Pfeiffer phenomenon occurred in 1894 when Richard Pfeiffer observed and recorded that the sera of guinea pigs which had recovered from experimental cholera contained some substance or substances which would lyse and kill Vibrio comma (cholera). He noted that when living vibrios were injected into a guinea pig previously infected with these organisms, these virulent cholera vibrios showed marked morphologic changes including loss of motility followed by a gradual granulation, swelling, and disappearance by lysis into the fluid. Jules Bordet (1895) reported that mammalian sera is capable of causing the dissolution or lysis of certain bacteria and that complement may be involved in the process. This can readily be demonstrated in vivo by injecting a suspension of Vibrio comma intraperitoneally into a guinea pig along with an anticholera serum which is devoid of complement as a result of having been heated to $56^{\circ}C$ for 30 minutes or by storing for a time. If peritoneal fluid is then drawn off with a hyperdermic syringe at intervals within an hour,

it is seen that the vibrios undergo progressive lysis and disappear from the fluid. In this case, the complement of the blood plasma of the animal acts with the injected antibody of the immune serum (Mackie, 1953). This same reaction can be produced in vitro when antibody, complement, magnesium and calcium ions are present and is the basis for measuring the bactericidal effect of serum by the photometric assay method.

The work of Metchnikoff (1905) and Bordet (1909) in the early 1900's proved that bacteriolysis occurred outside the body upon the addition of fresh peritoneal fluid or normal serum to heat inactivated ($56^{o}C$ for 30 minutes) immune serum. It was also found that anti-serum heated up to $70^{o}C$ for one hour produced the reaction when injected with the corresponding organism into the peritoneum of a normal animal. In summary then, when an animal is immunized against an organism, and antibody appears in its serum which is comparatively heat stable and resists a temperature of up to $70^{o}C$ for one hour. It cannot produce a destructive effect by itself but requires the presence of complement. Complement is not increased during the process of immunization.

In 1899 Bordet recorded his findings with regard to the specific action of natural agglutinins appearing in normal serum. He demonstrated that by absorbing normal horse serum with Vibrio comma and then removing the bacteria and attached agglutinins by

centrifugation, the serum lost its ability to agglutinate Vibrio comma but would still agglutinate typhoid bacilli as intensely as the unabsorbed serum. Muir and Browning (1908) recorded similar absorption experiments in order to determine the specificity of action of bactericidal activity in normal serum. They found that absorbing normal serum with increasing amounts of bacterial suspension produced first a diminution of the bactericidal action towards the homologous bacterium, and then a decrease in the effect of the serum on other bacteria.

The Neisser-Wechsberg effect was reported in 1901 to indicate the fact that bacteriolysins and bacteriocidins exhibit a prozone effect in the presence of excess antibodies as do agglutinins. That is, serum containing a high concentration of antibody against a gram negative bacillus is incapable of killing that strain in the presence of a normal amount of complement whereas the bactericidal effect can be demonstrated if the antiserum is diluted. Thus, in serum containing bactericidal antibody, there is an optimum amount of antibody which gives the greatest bactericidal effect with a given amount of complement. If this amount is exceeded there is first a reduction of killing and then a completed disappearance (Neisser and Wechsberg, 1901).

Pettersson (1926, 1927, 1928) classified the bactericidal agent of serum which kills or lyses gram negative bacilli as alpha lysin,

a term which is sometimes used today. Alpha lysin consists of two components including complement which acts along with a sensitizing agent analogous to an immune body.

In the 1930's Muir and Mackie made many significant contributions. Muir (1931) distinguished between bacteriolytic and bactericidal effects due to the antigen-antibody-complement system and concluded that only a few strains of bacteria are actually lysed as compared with many strains being killed. He further contributed additional information with regard to pour plate methods for bactericidal determinations. Mackie (1931, 1932) demonstrated the specificity of the killing action of normal serum by various absorption tests. He also studied and explained in great detail the mechanism of killing action of the antibody complement system. He presented several new techniques for determining the bactericidal effects of normal and immune serum using constant amounts of serum or whole blood with serial dilutions of live bacteria. He demonstrated that a graded series of culture dilutions made with a constant dilution factor provide what is in effect a logarithmic scale upon which the bactericidal power may be measured in terms of the numbers of dilutions exhibiting no bacterial growth. Miles and Misra (1938) applied Mackie's work to develop a surface viable plate count method which is frequently used today.

Recent Developments (1948-1963)

Muschel and/or Treffers (1948, 1952, 1954, and 1956) published first in partial reports and later in complete description a photometric assay method for quantitatively measuring the killing effect of antibody plus complement in both normal and immune sera. Using this technique they were able to quantitate the reacting components at a molecular level which has aided greatly in the advancement of knowledge concerning the mechanism of the killing action. They found that the quantitative properties of the bactericidal reaction parallel those of the hemolytic reaction. Different amounts of the four components of complement are active in the two systems, however, as is illustrated by the fact that bovine serum serves as a very efficient complement source in the bactericidal reaction and as a very ineffective source of complement in the hemolytic reaction.

Less antibody is necessary to sensitize susceptible bacteria to the killing action of complement than is required to produce agglutination or precipitation. The amount of antibody necessary to sensitize each bacterium to the killing action by complement was calculated to cover less than 1% (0.03 - 0.7%) of the bacterial cell surface. This is determined by the fact that in a typical experiment, 700-860 molecules of rabbit sera antibody and 15,000,000 molecules of guinea pig serum complement were required to kill one bacillus of

Salmonella typhosa at the 50% survival end point. Determinations were not made to indicate what per cent of the cell surface need be covered with antibody before a prozone or Neisser-Wechsberg effect might occur.

One advantage of the antibody complement bactericidal system in addition to its sensitivity, is that it is applicable to a large number of gram negative bacilli. Many genera including Vibrio, Salmonella, Shigella, Proteus, Hemophilus, and Brucella have been demonstrated as susceptible agents in this system (Muschel, 1959, 1960). A few non-susceptible strains have been found. Some strains of Paracolon ballerup, Salmonella typhimurium, and Salmonella paratyphosa C are completely insusceptible to both normal and immune serum under usual test conditions (Osawa, 1960; Muschel, 1960).

The bactericidal action of normal serum has been attributed to many causes including specific antibodies (Mackie, 1931, 1932; Landy et al., 1962), non-specific antibodies (Gordon and Carter, 1932), the properdin system (Wardlaw and Pillemer, 1956), and to the lysozyme system (Wardlaw, 1962). It has been studied extensively by Muschel using the photometric assay technique, demonstrating that the killing effect of normal serum requires the presence of a substance which may be regarded as an antibody. This normal serum antibody substance was found to be specific in

action since it can be selectively absorbed by its specific antigen. In the absence of this antibody, properdin and lysozyme were found completely lacking in bactericidal action (Osawa and Muschel, 1959). In the presence of this antibody, however, properdin slightly augmented or had no effect on the antibody titer while 0.1 mg of egg white lysozyme markedly enhanced the killing effect of the normal serum, increasing the antibody titer by as much as 50%. Contradictory evidence was obtained by (Wardlaw, 1962) in testing the bacteriolytic action of normal human serum on a rough strain of E. coli. He found the bacteriolytic process of normal serum to have no definite requirement for properdin or specific antibody. Rather, the lysis was dependent on temperature, ionic strength and pH, and on the combined action of complement and lysozyme.

The origin of antibody found in normal sera is not readily determined. Experiments by Muschel also indicated that the sera of 6-week-old germfree guinea pigs were comparable in bactericidal activity against Shigella dysenteriae and Salmonella typhosa strain 901, to the sera of guinea pigs of the same strain and age kept in a conventional environment. However, these germfree animals were not free from possible antigenic stimuli of dead bacteria which may have been present in sterile food and water nor were they free of possible passively transmitted maternal antibody.

Immune antibody is known to differ from normal serum antibody

only in its property of increased thermostability. It occurs in response to specific immunization. Because of the magnitude of dilutions which must be made before testing immune sera by the photometric assay test, factors in the serum other than antibody, such as lysozyme which influence the antibody titers of low dilutions of normal serum, are not present in effective concentrations to influence the antibody titers of highly diluted immune serum.

CHAPTER III

MATERIALS AND METHODS

Special Equipment Other Than Routine Bacteriological Equipment.

1. Photoelectric Colorimeter

   Any suitable photoelectric instrument capable of determining turbidity in units of optical density may be used. A Bausch and Lomb Spectronic 20 Colorimeter was used throughout this study. The wavelength should be adjusted for minimal absorption by the medium itself. This was 550 mu for brain heart infusion broth.

2. Screw cap pyrex glass test tubes of convenient size pre-selected for optical uniformity so that they can be used in the photoelectric colorimeter.

3. Refrigerated centrifuge which can be operated at speeds up to 8000 rpm.

Reagents

1. Complement. Guinea pig serum.
2. Magnesium saline diluent.
3. Test serum.
4. Brain heart infusion broth.
5. Blood agar plates.
6. Salmonella pullorum (Edwards strain).

Preparation of Reagents

Complement

Preparation of complement was the most complicated and time consuming part of the test. Commercial lyophilized guinea pig serum containing no preservative was used as a source of complement throughout the study. It was reconstituted with cold distilled water rather than with the diluent normally supplied because the latter often contained antibacterial substances such as boric acid which interfered with the test.

Since normal guinea pig serum contains natural bactericidal antibody for Salmonella pullorum, it must be absorbed before using as a source of complement in the test. Double absorption was found to be necessary most of the time, using the cells from a 250 ml overnight culture of Salmonella pullorum in brain heart infusion broth per single absorption per 10 ml of guinea pig serum. The factors which influenced the successes and failures will be discussed later.

The overnight broth culture of Salmonella pullorum was heat killed by submerging in a 60°C water bath for 1 hour. The killed bacterial suspension was then centrifuged and the supernatant broth discarded. Sterile magnesium saline diluent was then added and the bacteria washed three times using the same procedure.

Just before absorption, the lyophilized complement was reconstituted with cold distilled water and added to the washed bacterial

button of cells. The bacteria were gently suspended and allowed to stand in a $0^{\circ}C$ ice bath for 1 hour. The mixture was then centrifuged in a refrigerated centrifuge at $4^{\circ}C$ for 15 minutes at 6000-8000 rpm and the supernatant complement transferred to a second fresh bacterial button for further absorption. The same process was repeated and the supernatant complement sterilized by passage through a cold membrane filter (Millipore HA, pore size 4.5 u). The complement was ready for use and could be stored frozen for several weeks before using without significant loss of activity.

Diluent

The diluent used throughout the test was physiologic saline solution containing the optimal amount (50 ug/ml) of magnesium ion for the bactericidal action of serum. The diluent contained:

$MgCl_2.6\ H_2O$	0.063%
NaCl	0.85%

The diluent was sterilized by autoclaving at $121.6^{\circ}C$ under 15 lbs. pressure for 15 minutes and could be stored in the refrigerator for an indefinite length of time.

Test Serum

Each blood sample to be tested was allowed to clot at room temperature for 15 minutes to 2 hours and then centrifuged at room temperature to separate the serum. The serum was then sterilized by passage

through a membrane filter (Millipore HA, pore size 4.5 u), and stored frozen to preserve any thermolabile bactericidal antibodies which might be present.

Bacterial Inoculum

A standard strain of Salmonella pullorum obtained from Dr. Edwards at the Communicable Disease Center was used which exhibited the following characteristics as a gram negative, non-motile bacillus.

H_2S positive

TSI alkaline slant, acid butt, gas slight or variable

Lactose no reaction

Sucrose no reaction

Methyl Red positive

Voges Proskauer no reaction

Indol no reaction

Motility no reaction

Urea no reaction

Citrate no reaction

Antigenically, Salmonella pullorum is closely related to Salmonella typhosa as both belong to group D of the Kauffman-White scheme of classification. They share the common "O" antigens

9 and 12. Salmonella pullorum, does not contain a Vi antigen and being non-motile, has no "H" or flagellar antigen.

For the purpose of maintaining the organism in as smooth, virulent, and antigenically active a condition as possible, the Salmonella pullorum was passed through 12 successive mouse passages before starting this project and through several additional mouse passages during this study. The stock culture was then maintained on blood agar plates incubated at 38°C and stored in the refrigerator.

The bacterial inoculum used in the test consisted of an overnight (12 to 16 hour) subculture in brain heart infusion broth. Just before use, the culture density was standardized by adding sterile brain heart infusion broth to give a transmittance of 72% at 550 mu wavelength using a clear tube of brain heart infusion broth as a blank. The adjusted inoculum was then placed in a O°C ice bath to stop further growth during the pipetting.

Test Procedure

To enhance uniformity, to prevent deterioration of complement, and to prevent further growth of the inoculum during the pipetting process, all pipetting was done in an ice bath at O°C. The order for adding reagents as suggested by Muschel and Treffers is as follows: The serum first, then complement, then sufficient diluent

to bring all tubes to a constant volume of 1.7 ml, and finally the bacterial inoculum.

A preliminary test, covering a wide range of serum dilutions was often necessary to determine the approximate antibody concentration. For preliminary titration, the following dilutions might be used: 1-10, 1-100, 1-500, 1-1000, 1-5000, 1-10,000, and 1-20,000. See Table 1 for details. The proper serum dilution to be used in the actual test can be selected from the approximate titer obtained in the preliminary test.

The actual test makes use of four or more quantities of a single serum dilution calculated to fit in regular spaced intervals along a logarithmic scale. Suggested amounts are 1.35 ml, 0.9 ml, 0.6 ml, and 0.4 ml. See Table 1 for details.

Test and Control Tubes

A set of control tubes was included along with a set of serum test tubes in every preliminary and actual test run. Each control tube served a necessary function in determining the killing action of the test serum. All of these tubes (test and control) were read in a regular order as quickly as possible. Each tube, in order of reading, is listed below with its components and function.

1. Blank: Contained complement, diluent and broth. This tube was used for adjusting the photoelectric colorimeter to 100% transmittance

TABLE 1 PROCEDURES FOR PRELIMINARY AND ACTUAL TESTS USED IN THE BACTERICIDAL ASSAY

Preliminary Test (Reagents Listed in ml. in Order of Addition)

	Selected Serum Dilutions							Serum Control	Diluent Control	Compl. Control	Blank
	1-10	1-100	1-500	1-1000	1-5000	1-10,000	1-20,000	1-10			
Serum	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	-	-	-
Complement	0.3	0.3	0.3	0.3	0.3	0.3	0.3	-	-	0.3	0.3
Diluent	0.8	0.8	0.8	0.8	0.8	0.8	0.8	1.1	1.7	1.4	1.4
Bacterial inoculum	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	-
Broth	-	-	-	-	-	-	-	-	-	-	0.3

Actual Test (Reagents Listed in ml. in Order of Addition)

	Selected Serum Dilution (such as possibly 1:5000)				Serum Control 1:5000	Diluent Control	Complement Control	Blank
Serum	1.35	0.9	0.6	0.4	1.35	-	-	-
Complement	0.3	0.3	0.3	0.3	-	-	0.3	0.3
Diluent	0.05	0.5	0.8	1.0	0.35	1.7	1.4	1.4
Bacterial inoculum	0.3	0.3	0.3	0.3	0.3	0.3	0.3	-
Broth	-	-	-	-	-	-	-	0.3

at a wavelength of 550 mu. It represented the contents of each other tube before bacteria were added.

2. Serum Control: Contained serum (largest volume of test serum), diluent and bacteria. This tube determined the killing action of the largest volume of test serum using no other source of complement. In low dilutions, the serum control indicated the effectiveness and the quantity of complement present in the serum of the test animal since without an excess supply of one component active in the bactericidal reaction it proved a limiting factor for measuring another. In high dilutions the serum control served as an indicator of possible contamination of the test serum by toxic agents or bacteria since an irregular growth curve resulted under these circumstances.

3. Diluent Control: Contained diluent and bacteria. This tube represented maximum survival of bacteria under conditions of the bactericidal test. This tube contained no serum or inhibitors.

4. Complement Control: Contained complement, diluent and bacteria. This tube measured the killing action of the absorbed guinea pig complement. Standards of the test required survival of bacteria in this control to be at least 75% of that in the diluent control tube.

5. Serum Test: Contained test serum, complement, diluent and bacteria. This tube contained the complete antibody plus

complement bactericidal system and the amount of killing which occurs was dependent upon the amount of antibody contained in the test serum.

Reaction Time

Immediately after adding all ingredients in either the preliminary or actual test, the tubes were gently mixed and placed in a 37°C water bath for 1 hour. During this period, the antibody, if present, was given an opportunity to act with the complement in killing the viable bacteria contained in the inoculum.

Growth Time

At the end of the reaction time, 5 ml of brain heart infusion broth was added as rapidly as possible to each tube. The broth was anti-complementary and terminated the bactericidal action by destroying the complement, by diluting the reactants, and by providing sufficient nutrients for the viable bacteria to multiply. The tubes were gently mixed and the initial readings of density taken as quickly as possible using the photoelectric colorimeter. This reading at 0 hours permitted corrections to be made for irregularities such as hemolyzed serum and irregular tubes. The tubes were then placed in a 37°C water bath. At this time the growth time stage of the photometric assay began.

The photoelectric colorimeter used during this study exhibited maximum sensitivity between 10 and 90% transmittance. Turbidometric

growth curves as well as plate counts indicated, however, that the bacterial growth slowed down and was no longer logarithmic when the photoelectric colorimeter read about 35% transmittance. Consequently, an incubation period was selected such that the diluent and complement control tubes still had a transmittance greater than 35% and yet had grown enough to demonstrate that the bacteria were in an active logarithmic phase of growth (less than 66% transmittance). In the Salmonella pullorum system an incubation period of 4 and 5 hours met these specifications and was therefore selected as the time for measuring the density of each tube.

For convenience, readings were taken in terms of transmittance on the photoelectric colorimeter and converted to units optical density for calculation. The apparent per cent survival was then computed from the optical density and an average of the 4 hour and 5 hours figure used for determining the final serum titer.

Calculation of Results

1. The bactericidal effect of antibody plus complement was expressed in terms of apparent per cent survival of the exposed live bacilli. For any given tubes this figure was equal to the optical density X 100 divided by the optical density of the control tube containing no serum (antibody). Thus:

$$\text{Apparent \% Survival of Test Serum} = \frac{\text{Test Serum (O. D.) X 100}}{\text{Diluent Control (O. D.)}}$$

Likewise, the bactericidal effect of complement alone (without antibody) could be determined:

$$\text{Apparent \% Survival of Complement} = \frac{\text{Complement Control (O. D.) X 100}}{\text{Diluent Control (O. D.)}}$$

2. The apparent per cent survival of each serum dilution was then plotted versus the logarithm of the amount of serum on either probit or semi-logarithm graph paper.

3. That amount of serum which would result in a 50% survival of the reactant bacteria was then determined from the graph. This value could also be expressed in another way as serum titer (the dilution of serum at which 1.0 ml produced the 50% survival end point).

Representation of Data by Graph

The experimental results could be accurately represented on either semi-logarithmic or probit graph paper. On semi-logarithmic graph paper, the plotting of apparent per cent survival against the logarithm of the corresponding amount or dilution of serum resulted in a sigmoidal curve. Such a graph was adequate for determining the 50% survival end point of a given amount of test serum but was limited for statistical evaluation of the process. Representation of the same data on probit graph paper produced a straight line and was much more suitable for statistical analysis. It was, therefore, highly recommended by Muschel and Treffers (1956).

The probit slope (also referred to as response slope) was defined as the increase in probit value per unit increase in serum volume. To compute the slope, the serum volumes were first converted to logarithmic units. For example, 1.35 and 0.9 ml of a given serum dilution were equal to a logarithmic difference of 0.18 logarithmic units. The corresponding increase in probit value could then be read off of the probit graph.

Other advantages of probit analysis which were pertinent to this study include:

1. The standard deviation of distribution was equal to the reciprocal of the slope.

2. A change of slope indicated a change in the efficiency of action which effected the distribution.

3. If two curves were parallel, the relative efficiency values (potencies) could be given by the displacement at any level of growth inhibition (survival). If the lines were not parallel, the potencies varied with each level of growth inhibition examined.

Apparent Per Cent Survival as True Per Cent Survival

For practical purposes, the apparent per cent survival was a good indication of the number of bacteria surviving the reaction time. This figure was not, however, quantitatively proportional to the survival as could be demonstrated by inoculating a series of broth tubes with graded amounts of seed bacteria and incubating. The resulting optical densities

at any given time did not lie on a straight line with a 45° slope but instead formed a curve with disproportionately low readings for the higher concentrations of inocula. This slowing down of growth in the more heavily inoculated tubes was due to an accumulation of acid or other metabolic products, a deficiency of nutrients, and lack of space. The apparent survival curve could be corrected for this inhibitory effect but was not deemed necessary for this study in view of the following facts:

1. The apparent per cent growth was a reproducible experimental quantity.

2. The relative values of two sera were not changed by this correction and consequently for comparative purposes the apparent and true survival per cents were of equal value.

3. In order to perform this correction accurately, many series of growth curves needed to be conducted and a standard deviation computed. Therefore, in view of the additional time and effort required and the small amount of pertinent information gained with regard to this study, it was decided to omit this factor.

Rabbit Immunization Curve

A rabbit immunization program was selected as a means of testing the photometric assay technique for measuring Salmonella pullorum antibody. Three rabbits were selected for immunization and identified through the study as Rabbit #1, Rabbit #2, and Rabbit #3. Rabbits #1

and #3 were both young females about 6 weeks of age and #2, also a female, about 8 months of age.

Immunization Schedule

The immunization schedule provided that every four days, for four successive immunizations, doses were administered intramuscularly, each rabbit receiving 0.5 ml, 1.0 ml, 1.5 ml, and 2.0 ml. The antigen was prepared by heat killing (60°C for 1 hour) an overnight brain heart infusion broth culture of Salmonella pullorum. The bacterial broth suspension was then centrifuged and the bacteria washed twice in sterile physiological saline. The concentration was adjusted to that of a $BaSO_4$ Nephelometer #6, merthiolate added to give a final concentration of 1:10,000 and an equal volume of Alginate adjuvant added. The antigen was stored in the refrigerator and used for all immunization doses. One rabbit (#1) was given a single booster dose of 1 ml antigen without adjuvant seventy-three days after the fourth immunization dose.

Bleeding Schedule

The rabbits were bled two days before receiving the primary immunization series to obtain a pre-immunization base titer and then at four day intervals spaced to occur two days after each injection. As the serum titers began to decrease, the intervals between bleedings were lengthened. Rabbit #1 was bled at two and six days after its single booster immunization.

Technique of Bleeding

Bleeding of rabbits from the marginal ear vein presented problems and during the first portion of the study the very effective but often dangerous cardiac puncture was used. Unfortunately, one of the rabbits (the most responsive to immunization) died from trauma of a cardiac puncture before an equally efficient method was substituted.

The new method consisted of removing all hair from the marginal ear vein with a dipilatory such as Nair. The ear was then cleansed with alcohol and a small distal section of the vein selected. The top surface of the section was swabbed with mineral oil to prevent clotting and the other side of the ear section swabbed with xylol to stimulate bleeding. A tiny prick slit was then made with a lancet in the marginal vein and a tube held to collect the blood. This method of bleeding was very effective and also not injurious to the rabbit.

Agglutination Tests

For the purpose of comparing the bactericidal antibody and agglutinin antibody immune responses, agglutination tests were performed on as many of the serum samples as possible. An overnight brain heart infusion broth culture of Salmonella pullorum was centrifuged and the bacterial button washed two times in 0.3% formalin physiological saline. For storage the antigen was adjusted to a $BaSO_4$ Nephelometer #9.

Before use it was adjusted to that of a Nephelometer #3 and used immediately.

A thirteen tube, two fold serial dilution scheme was used such that tube #1 contained a 1:8 dilution and all tubes had a final volume of 1 ml after 0.5 ml of antigen was added.

Two control tubes were also included such that each contained 0.5 ml physiological saline and 0.5 ml antigen. These controls served as an indicator for spontaneous agglutination.

After mixing, the tubes were placed in a 48-50^{o}C water bath, incubated for 16 to 18 hours and examined for agglutination. The agglutination titer was expressed as the reciprocal of the greatest dilution of serum giving a visible agglutination reaction.

CHAPTER IV

RESULTS AND DISCUSSION

Tables and Graphs

Bactericidal assays and agglutination tests were performed on the sera of three rabbits before immunizing with Salmonella pullorum and after immunization at regular intervals according to the procedures given in chapter three. The bactericidal assays were conducted separately for each bleeding within one month of obtaining blood. Agglutination tests were performed at the end of the immunization program simultaneously on all remaining serum samples of rabbit #3 and then in a second batch on all remaining serum samples of rabbits #1 and #2. A summary of these results is contained in table 2 and graphs 1 and 2ABC.

Table 2 includes the bactericidal antibody and agglutinin titers, their ratios, and the response slope from each bactericidal assay listed for each rabbit with the corresponding dates of immunization and bleeding. Graph 1 contains the bactericidal titer of each rabbit plotted against the date of bleeding. Graphs 2ABC contain the bactericidal antibody and agglutinin titers for each rabbit plotted against the date of bleeding and demonstrate the sensitivities of the two test methods in their proper perspectives.

Table 2

SUMMARY OF RESULTS

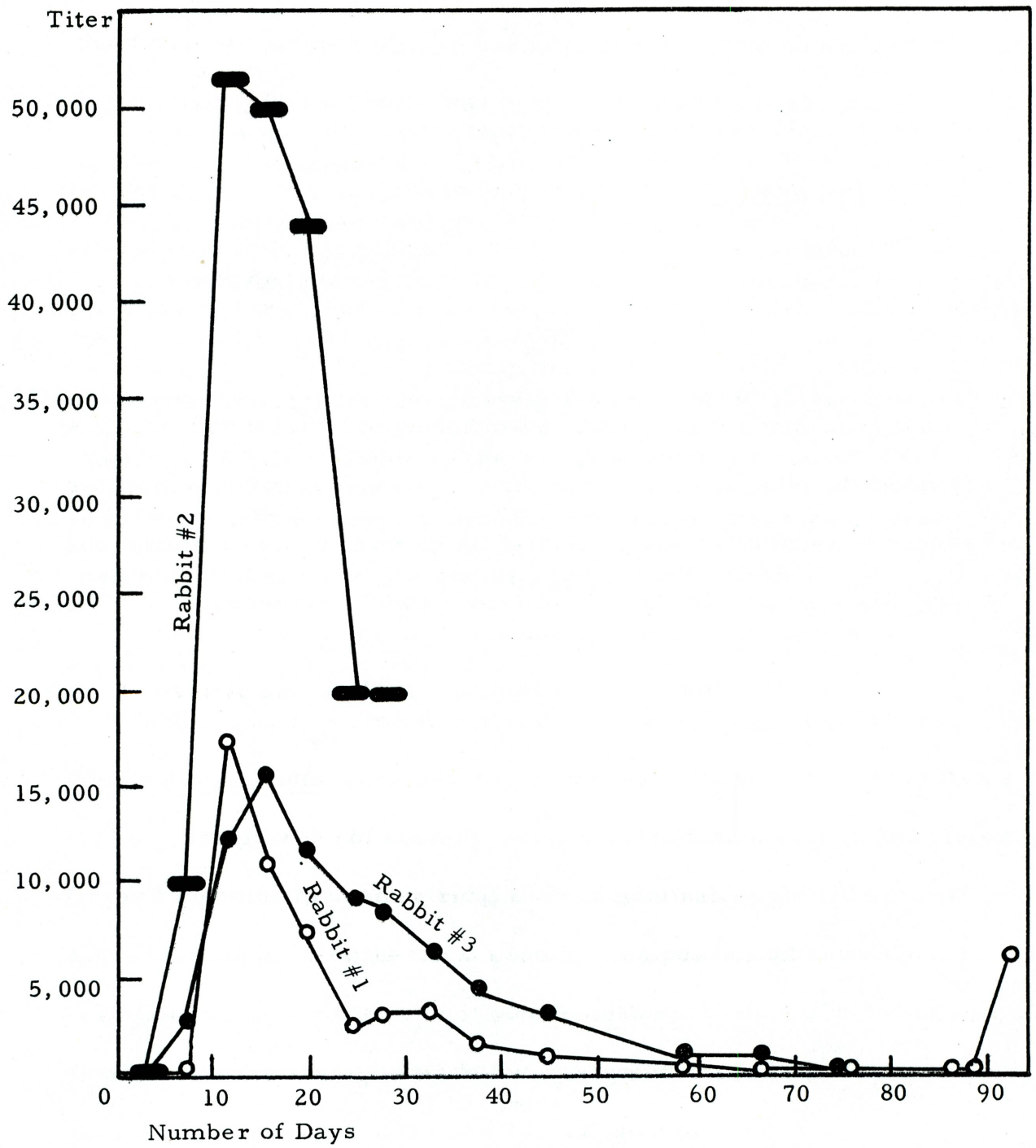
(Bactericidal and Agglutinating Titers and Response Slopes of Rabbits #1, #2, and #3)

Day	Inoc. (ml)	Bleeding	Rabbit #1 Serum Titers				Rabbit #2 Serum Titers				Rabbit #3 Serum Titers			
			Agg'l	Bact.	Ratio	Slope	Agg'l	Bact.	Ratio	Slope	Agg'l	Bact.	Ratio	Slope
0		1st	neg	3		1.5	neg	10		2.0	neg	2		.91
2	.5													
4		2nd	NSQ*	2		1.3	NSQ	14		2.2	NSQ	3		1.4
6	1.0													
8		3rd	16	395	25	2.8	512	10,000	20	2.6	64	2,940	5	2.4
10	1.5													
12		4th	128	17,540	137	3.2	2048	51,720	25	3.2	1,024	12,500	12	3.2
14	2.0													
16		5th	128	11,110	87	2.7	8192	50,000	6	2.7	1,024	15,960	16	3.6
20		6th	256	7,410	29	2.3	8192	44,440	5	3.0	256	11,905	5	2.3
25		7th	256	2,780	11	2.0	8192	20,000	2	2.4	512	9,300	2	2.7
28		8th	128	3,125	24	2.6	2048	20,000	10	2.8	512	5,880	11	2.8
33		9th	128	3,450	27	2.5					256	6,670	26	2.3
38		10th	128	1,905	15	2.5					256	4,650	18	3.0
45		11th	128	1,160	9	2.1					128	3,510	27	2.4
59		12th	64	890	14	2.4					64	1,430	22	2.4
67		13th	64	640	12	2.0					64	1,110	10	1.9
76		14th	64	280	5	2.3					16	280	17	2.6
87	1.0	15th	64	220	3	1.9								
89		16th	32	120	4	1.3								
93		17th	1,024	6,190	6	2.0								

*Non-sufficient quantity to test.

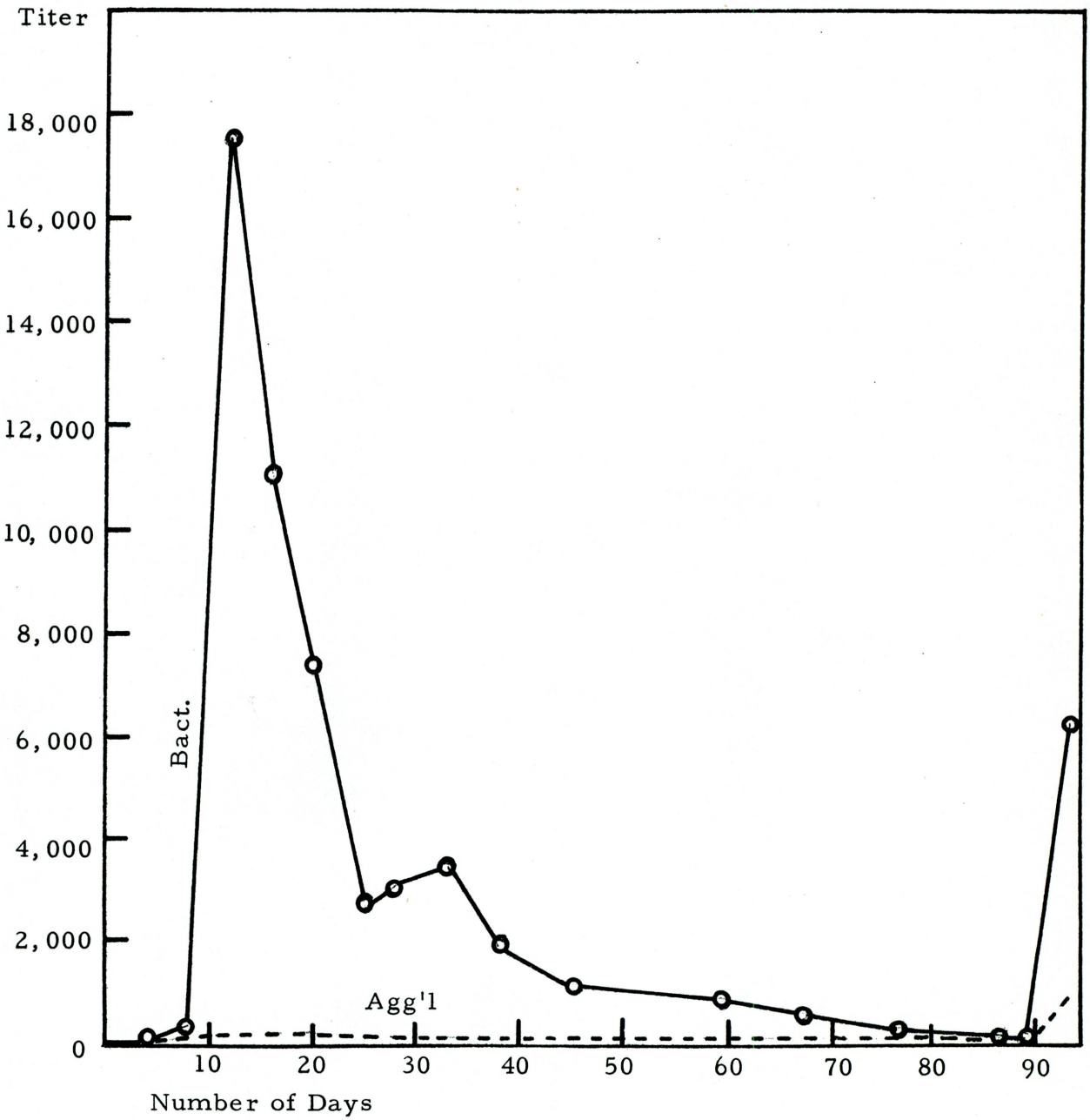
GRAPH 1

Antibody Responses of Rabbits #1, #2, and #3 (Bactericidal)



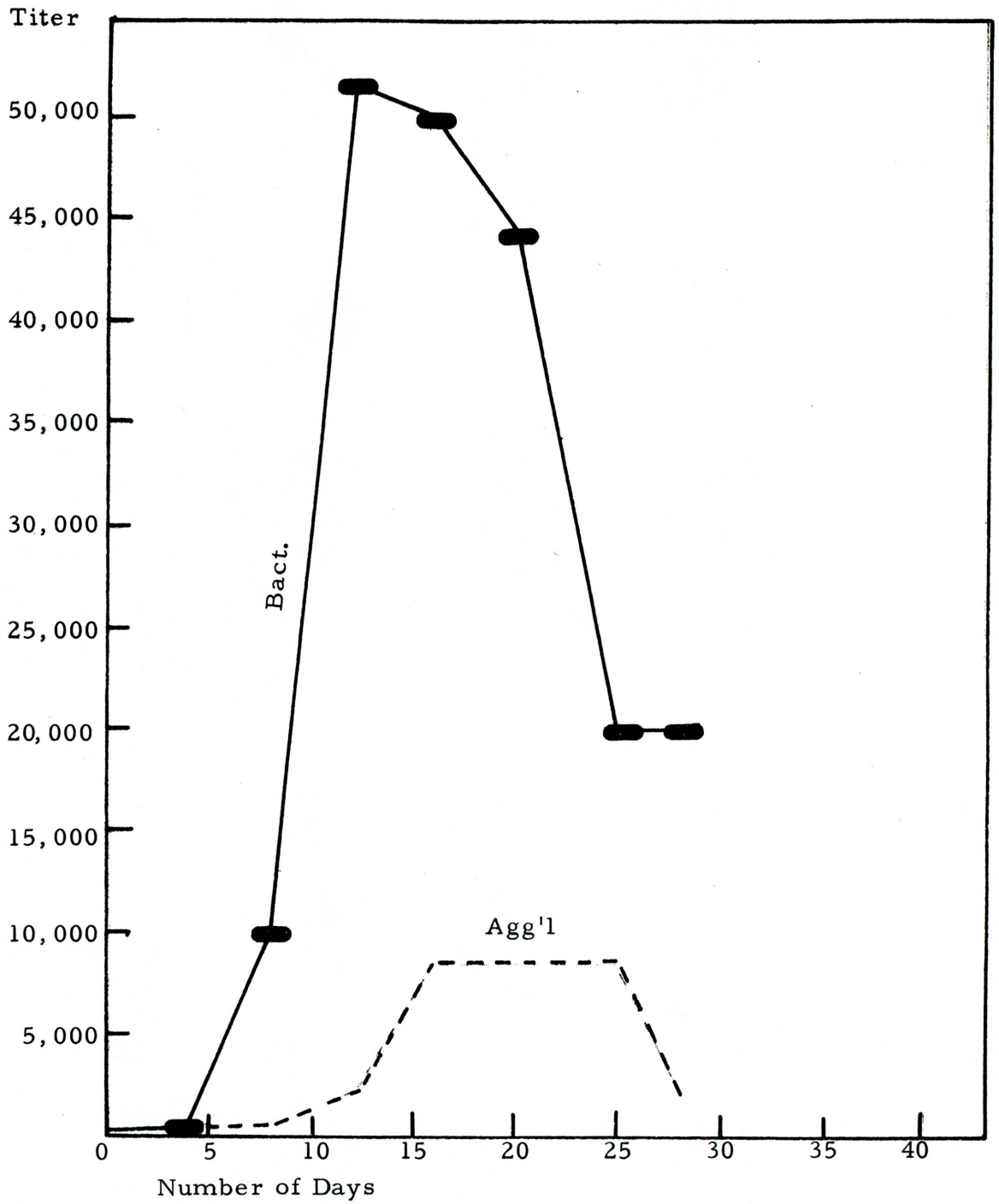
GRAPH 2A

Antibody Responses of Rabbit #1 (Agglutinating and Bactericidal)



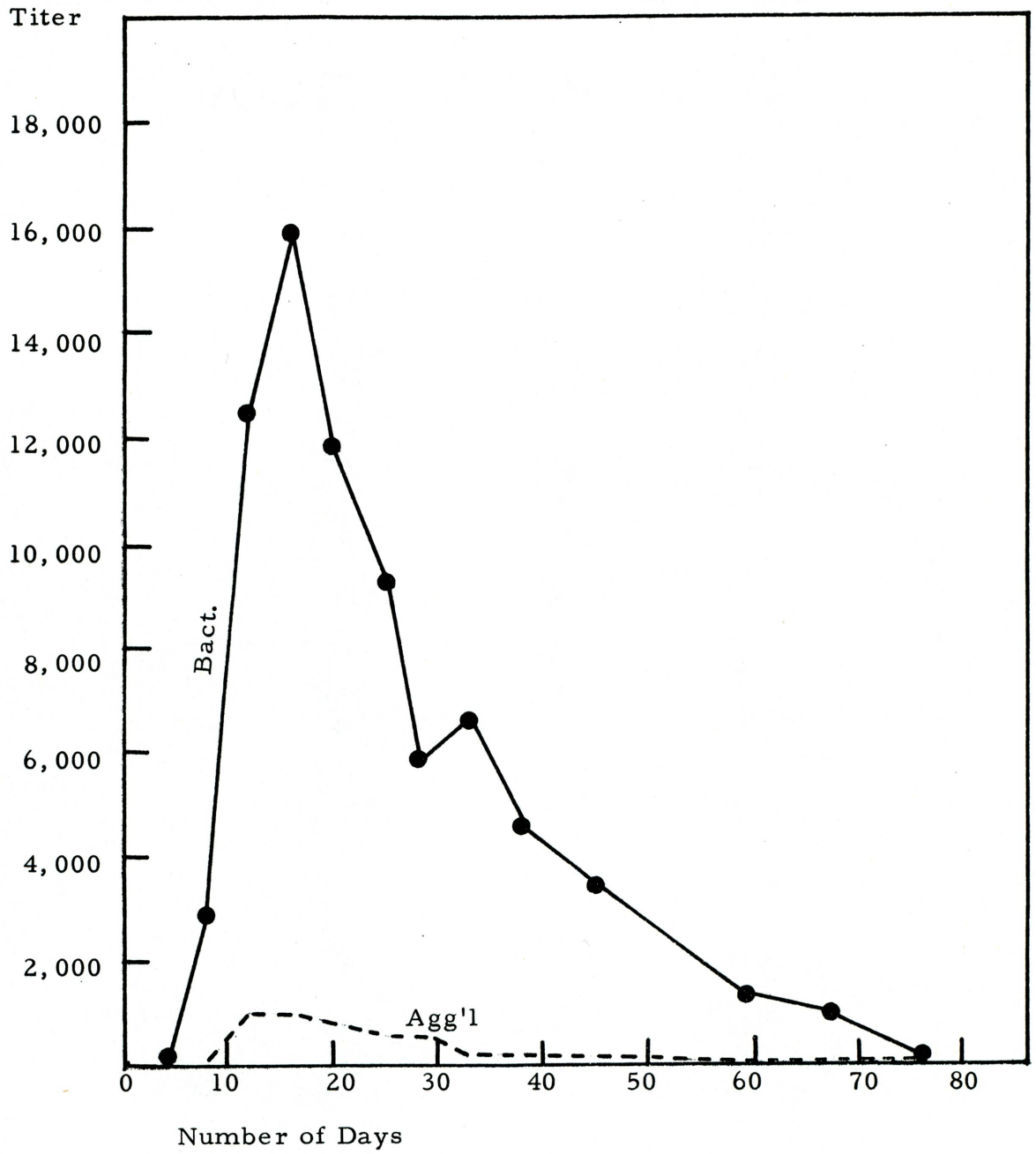
Graph 2B

Antibody Responses of Rabbit #2 (Agglutinating and Bactericidal)



GRAPH 2C

Antibody Responses of Rabbit #3 (Agglutinating and Bactericidal)



Tables 3-19 give the detailed data used in determining each bactericidal titer. Each table is accompanied by its corresponding graph from which the 50% survival end point is determined. These tables and graphs are numbered as follows:

First Bleeding	Table 3 and Graph 3
Second Bleeding	Table 4 and Graph 4
Third Bleeding	Table 5 and Graph 5
Fourth Bleeding	Table 6 and Graph 6
Fifth Bleeding	Table 7 and Graph 7
Sixth Bleeding	Table 8 and Graph 8
Seventh Bleeding	Table 9 and Graph 9
Eighth Bleeding	Table 10 and Graph 10
Ninth Bleeding	Table 11 and Graph 11
Tenth Bleeding	Table 12 and Graph 12
Eleventh Bleeding	Table 13 and Graph 13
Twelfth Bleeding	Table 14 and Graph 14
Thirteenth Bleeding	Table 15 and Graph 15
Fourteenth Bleeding	Table 16 and Graph 16
Fifteenth Bleeding	Table 17 and Graph 17
Sixteenth Bleeding	Table 18 and Graph 18
Seventeenth Bleeding	Table 19 and Graph 19

Table 3

BACTERICIDAL TITERS OF RABBIT SERA AT FIRST BLEEDING
(Preimmunization)

		Rabbit #1 Serum (1:5)				Rabbit #2 Serum (1:5)				Rabbit #3 Serum (1:5)				Comp.	Dil.
Ml diluted serum		1.35	0.9	0.6	0.4	1.35	0.9	0.6	0.4	1.35	0.9	0.6	0.4	--	--
% Trans. at 0 hour		100	100	100	100	100	100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	79	79	76	74	91	89	83	78	78	76	74	72	65	50
	Optical density	.105	.105	.12	.13	.04	.055	.085	.11	.11	.12	.13	.14	.19	.3
	Apparent % surv.	55	55	63	68	21	29	45	58	58	63	68	74		
5 hr. incub.	% Transmit.	64	64	61	59	83	80	73	65	65	62	59	58	49	40
	Optical density	.195	.195	.215	.23	.085	.1	.135	.19	.19	.21	.23	.24	.31	.4
	Apparent % surv.	63	63	69	74	27	32	44	61	61	68	74	78		
Aver. appt. % surv.		59	59	66	71	24	30	44	60	60	66	71	76		
50% Surv. end point		1.6 ml of a 1:5 dilution				0.52 ml of a 1:5 dilution				2.6 ml of a 1:5 dilution					
Serum titer*		3.1				9.6				1.9					

*Dilution of serum at which 1.0 ml produces 50% survival end point

Complement 70.5% surv.

GRAPH 3

BACTERICIDAL TITERS AT FIRST BLEEDING

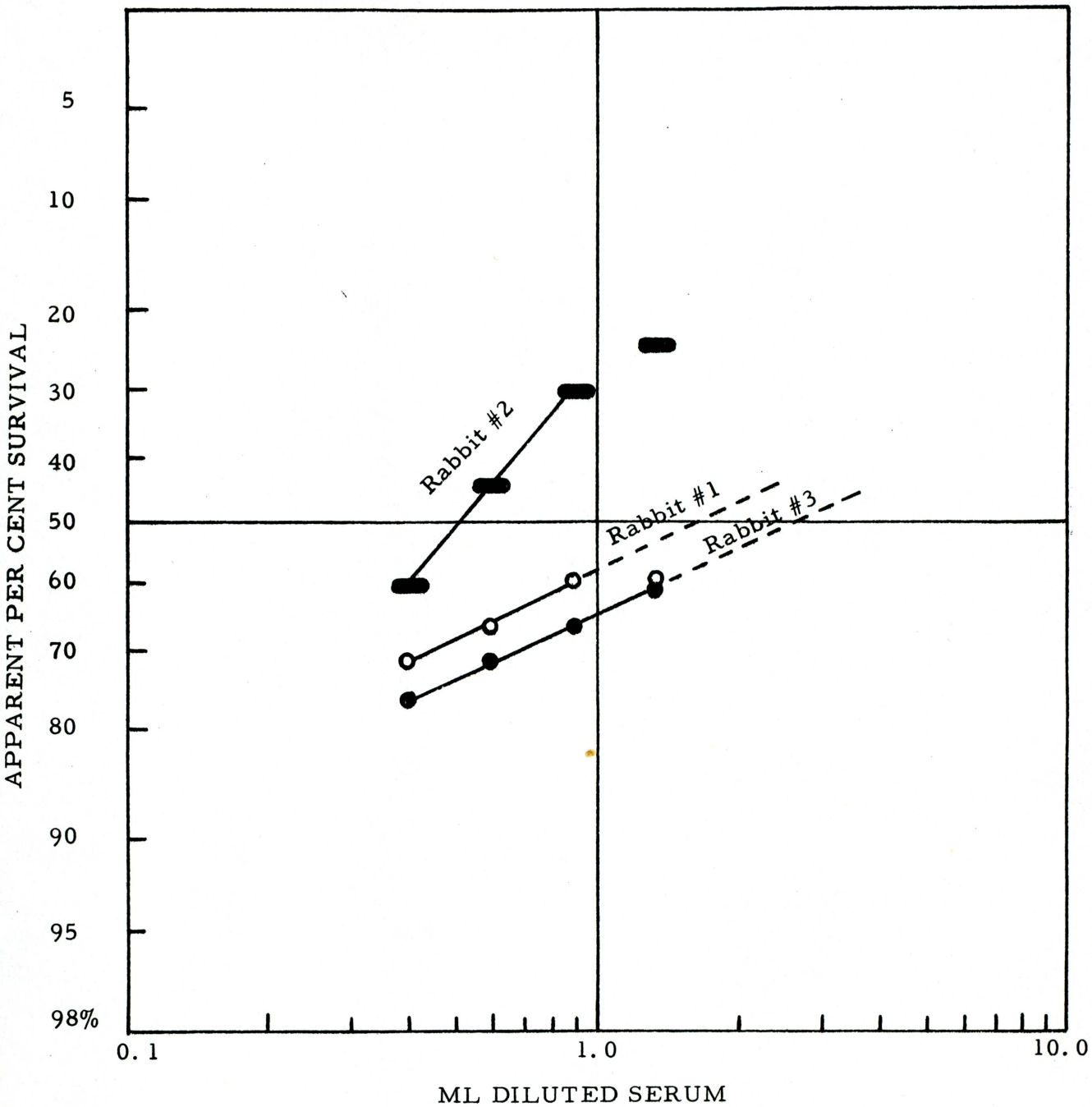


Table 4

BACTERICIDAL TITERS OF RABBIT SERA AT SECOND BLEEDING
(Two days after initial injection)

		Rabbit #1 Serum (1:5)				Rabbit #2 Serum (1:9)				Rabbit #3 Serum (1:6)				Comp.	Dil.
Ml diluted serum		1.35	0.9	0.6	0.4	1.35	0.9	0.6	0.4	1.35	0.9	0.6	0.4	---	--
% Trans. at 0 hour		100	100	100	100	100	100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	82	79	78	76	92	90	85	81	84	81	79	77	71.5	55.5
	Optical density	.09	.105	.11	.12	.035	.05	.075	.095	.08	.095	.105	.115	.145	.255
	Apparent % surv.	62	72	76	83	24	34	52	66	55	66	72	79		
5 hr incub.	% Transmit.	73	69	67	65	85	82	75	70	73	71	67	65	59	42.5
	Optical density	.135	.165	.175	.19	.075	.09	.125	.16	.135	.15	.175	.19	.23	.375
	Apparent % surv.	59	72	76	83	33	39	54	70	59	65	76	83		
Aver. appt. % surv.		62	72	76	83	28	38	53	68	57	66	74	81		
50% Surv. end point		2.2 ml of a 1:5 dil.				0.65 ml of a 1:9 dil				1.78 ml of a 1:6 dil.					
Serum titer*		2.3				13.8				3.4					

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 59% survival

Graph 4

BACTERICIDAL TITERS AT SECOND BLEEDING

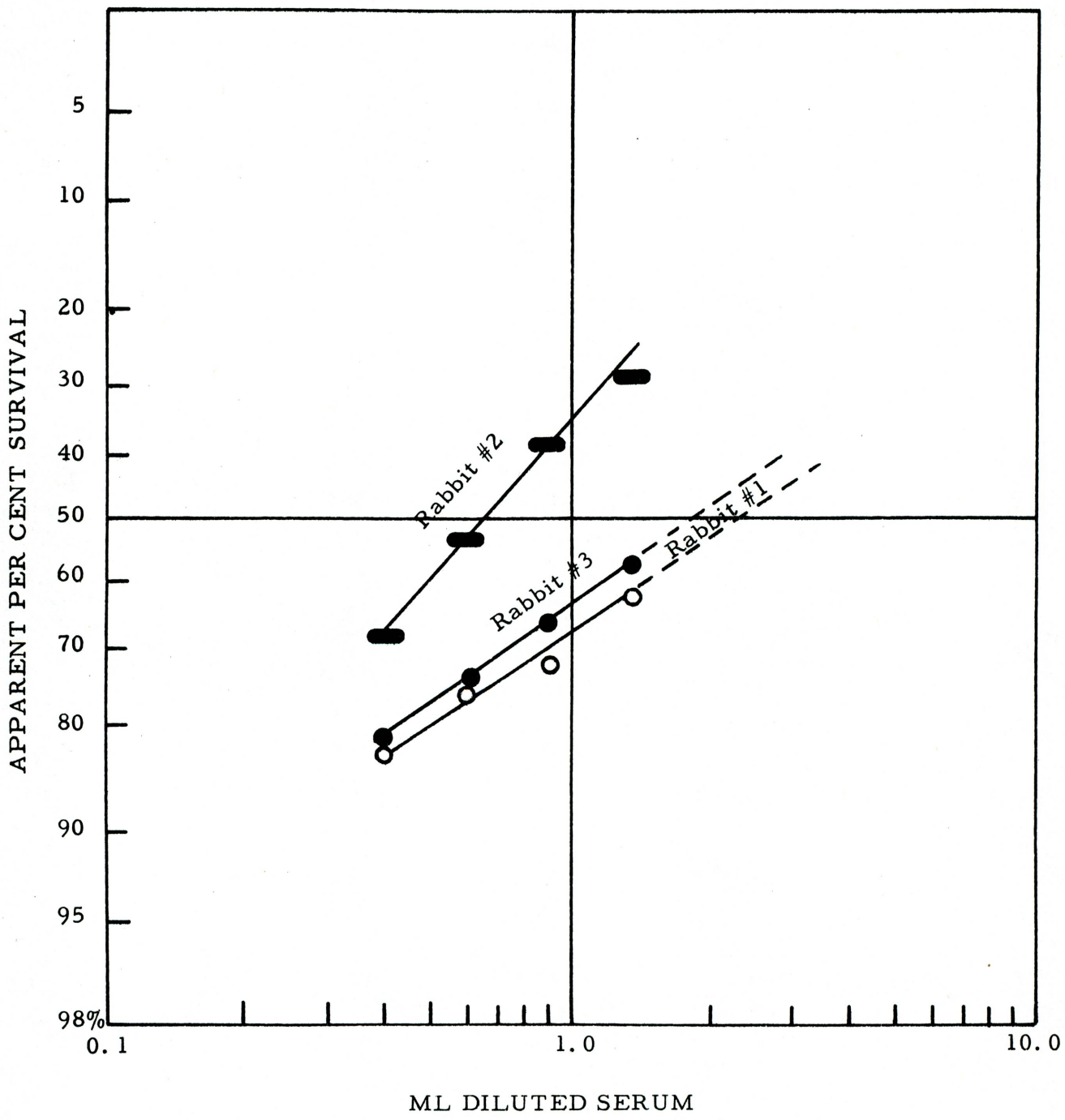


Table 5

BACTERICIDAL TITER OF RABBIT SERUM AT THIRD BLEEDING
(Six days after initial injection)

		Rabbit #1 Serum (1:150)					Comp.	Diluent
Ml diluted serum		1.35	0.9	0.6	0.4	0.1	---	---
% Trans. at 0 hour		100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	69	92	92	86	82	70	68
	Optical density	.165	.035	.035	.07	.09	.16	.17
	Apparent % surv.	100	22	22	44	56		
5 hr. incub.	% Transmit.	55	84	83	75	60	56	51
	Optical density	.26	.08	.085	.125	.22	.25	.295
	Apparent % surv.	100	32	34	50	88		
Aver. appt. % surv.		100	27	28	47	72		
50% surv. end point		0.38 ml of a 1:150 dilution						
Serum titer*		395						

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 90% survival

Table 5

BACTERICIDAL TITERS OF RABBIT SERA AT THIRD BLEEDING
(Six days after initial injection)

		Rabbit #2 Serum (1:5000)							Rabbit #3 Serum (1:500)					Comp.	Dil.
Ml diluted serum		1.5	1.35	0.9	0.6	0.4	0.25	0.1	1.35	0.9	0.6	0.4	0.1	---	--
% Trans. at 0 hour		100	100	100	100	100	100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	94	95	94	91	82	71	61	96	96	94	93	69	58	56
	Optical density	.025	.02	.025	.04	.09	.15	.215	.017	.017	.025	.03	.165	.24	.25
	Apparent % surv.	10	8	10	17	38	62	90	7	7	10	12	69		
5 hr. incub.	% Transmit.	90	89	84	72	49	60	49	91	92	89	87	56	44	41.5
	Optical density	.05	.055	.08	.14	.31	.22	.31	.04	.035	.055	.065	.25	.36	.385
	Apparent % surv.	14	14	15	22	39	61	86	11	9	15	18	70		
Aver. appt. % surv.		12	11	12	20	38	62	88	9	8	12	15	70		
50% surv. end point		0.50 ml of a 1:5000 dilution							0.17 ml of a 1:500 dilution						
Serum titer*		10,000							2941						

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 90% survival

GRAPH 5

BACTERICIDAL TITERS AT THIRD BLEEDING

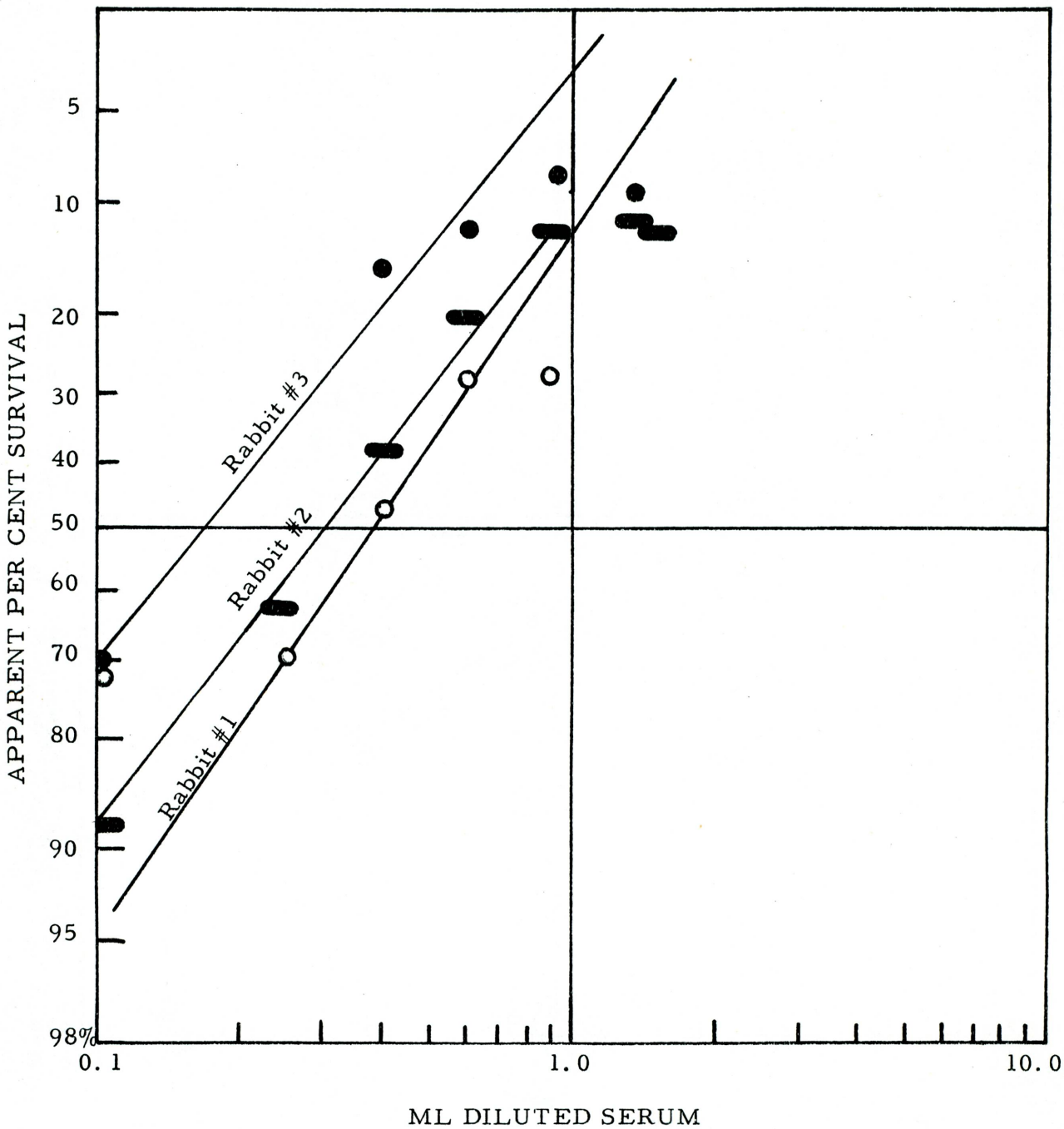


Table 6

BACTERICIDAL TITERS OF RABBIT SERA AT FOURTH BLEEDING
(Ten days after initial injection)

		Rabbit #1 Serum (1:500)						Rabbit #2 Serum (1:30,000)				Comp.	Diluent
Ml diluted serum		1.35	0.9	0.6	0.4	0.3	0.1	1.35	0.9	0.6	0.4	---	---
% Trans. at 0 hour		100	100	100	100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	96	96	95	91	83	70	96	91	83	77	67.5	62.5
4 hr. incub.	Optical density	.017	.017	.02	.04	.085	.16	.017	.04	.085	.115	.172	.205
4 hr. incub.	Apparent % surv.	10	10	12	23	49	93	10	23	49	67		
5 hr. incub.	% Transmit.	92	90	90	84	72	58	92	84	75	67	55	49
5 hr. incub.	Optical density	.035	.05	.05	.08	.14	.24	.035	.08	.125	.175	.26	.31
5 hr. incub.	Apparent % surv.	13	19	19	31	54	92	13	31	48	67		
Aver. appt. % surv.		12	15	16	27	52	92	12	27	48	67		
50% surv. end point		0.285 ml of a 1:5000 dilution						0.58 ml of a 1:30,000 dilution					
Serum titer*		17,544						51,724					

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 74.5% survival

Table 6

BACTERICIDAL TITER OF RABBIT SERUM AT FOURTH BLEEDING
(Ten days after initial injection)

		Rabbit #3 Serum (1:5000)				Comp.	Diluent
Ml diluted serum		1.35	0.9	0.6	0.4	---	---
% Trans. at 0 hrs.		100	100	100	100	100	100
4 hr. incub.	% Transmit.	94	95	91	82	65.5	57
4 hr. incub.	Optical density	.025	.02	.04	.09	.185	.245
4 hr. incub.	Apparent % surv.	14	11	22	49		
5 hr. incub.	% Transmit.	88	91	82	72	54	43
5 hr. incub.	Optical density	.06	.04	.09	.14	.27	.37
5 hr. incub.	Apparent % surv.	22	15	33	52		
Aver. appt. % surv.		18	13	28	50		
50% surv. end point		0.40 ml of a 1:5000 dilution					
Serum titer*		12,500					

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 74.5% survival

GRAPH 6

BACTERICIDAL TITERS AT FOURTH BLEEDING

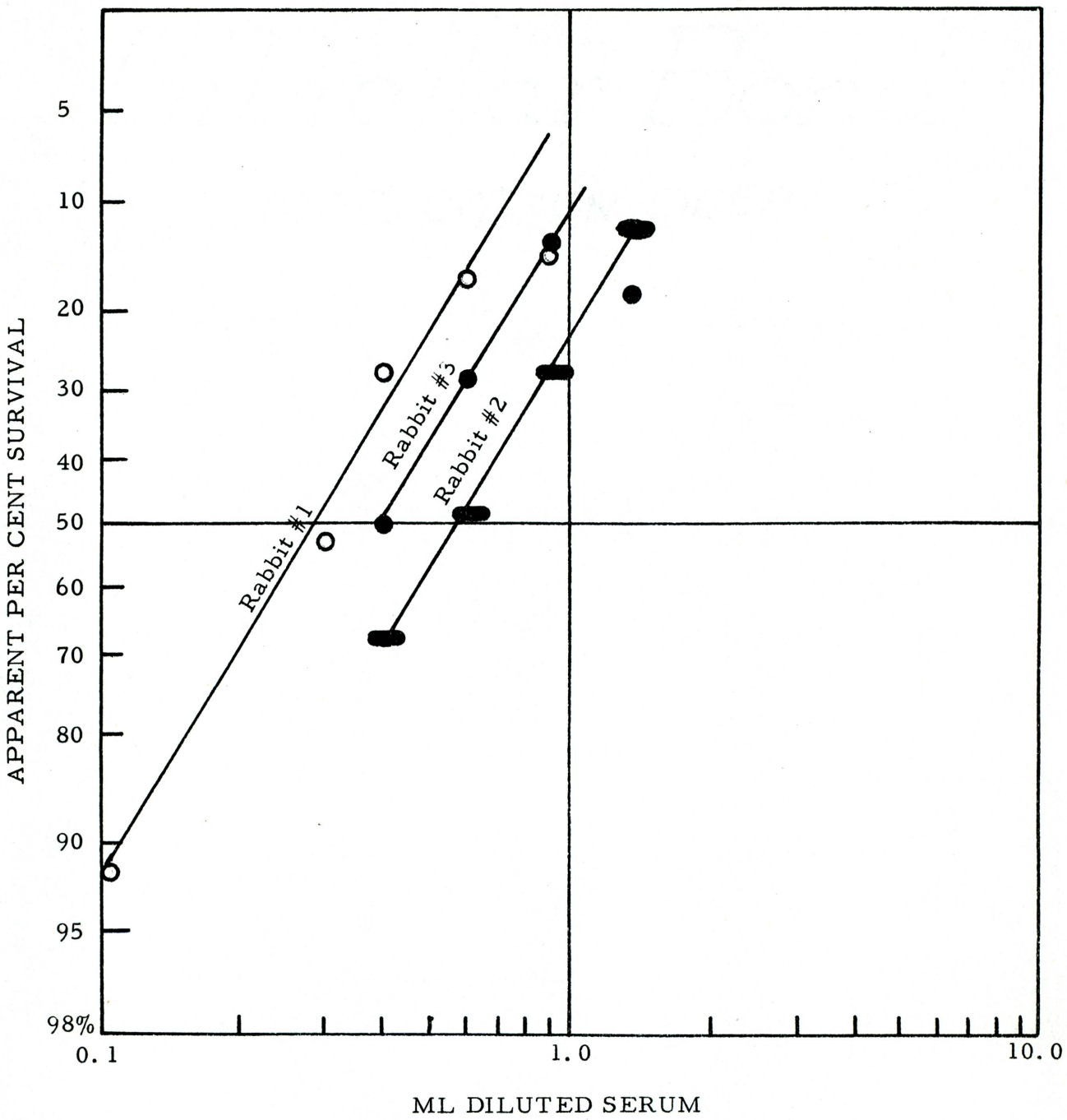


Table 7

BACTERICIDAL TITERS OF RABBIT SERA AT FIFTH BLEEDING
(Fourteen days after initial injection)

		Rabbit #1 Serum (1:10,000)					Comp.	Dil.	Rabbit #2 Serum (1:60,000)				Comp.	Dil.
Ml diluted serum		1.35	0.9	0.6	0.4	0.1	---	--	1.35	0.9	0.6	0.4	---	---
% Trans. at 0 hours		100	100	100	100	100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	91	85	81	76	73	70.5	63.5	85	79	74	70	67.5	62.5
	Optical density	.04	.075	.095	.12	.135	.155	.198	.075	.105	.13	.16	.172	.205
	Apparent % surv.	26	48	61	77	87			44	61	76	93		
5 hr. incub.	% Transmit.	84	76	69	64	64	59.5	51.5	76	67	61	57	55	49
	Optical density	.08	.12	.165	.195	.195	.225	.292	.12	.175	.215	.245	.26	.31
	Apparent % surv.	36	53	73	87	87			46	67	83	94		
Aver. appt. % surv.		31	50	67	82	87			45	64	80	94		
50% surv. end point		0.9 ml of a 1:10,000 dilution							1.2 ml of a 1:60,000 dil.					
Serum titer*		11,111							50,000					

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 84% survival

Table 7

BACTERICIDAL TITERS OF RABBIT SERA AT FIFTH BLEEDING
(Fourteen days after initial injection)

		Rabbit #3 Serum (1:15,000)					Comp.	Diluent
Ml diluted serum		1.35	0.9	0.6	0.4	0.1	---	---
% Trans. at 0 hours		100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	90	81	75	69	67	66.5	60.5
	Optical density	.05	.095	.125	.165	.175	.178	.218
	Apparent % surv.	28	53	70	93	98		
5 hr. incub.	% Transmit.	81	65	61	54	52	52.5	46.5
	Optical density	.095	.19	.215	.27	.29	.285	.335
	Apparent % surv.	33	67	75	95	100		
Aver. appt. % surv.		30	60	72	94	99		
50% surv. end point		0.94 ml of a 1:15,000 dilution						
Serum titer*		15,957						

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 84% survival

GRAPH 7

BACTERICIDAL TITERS AT FIFTH BLEEDING

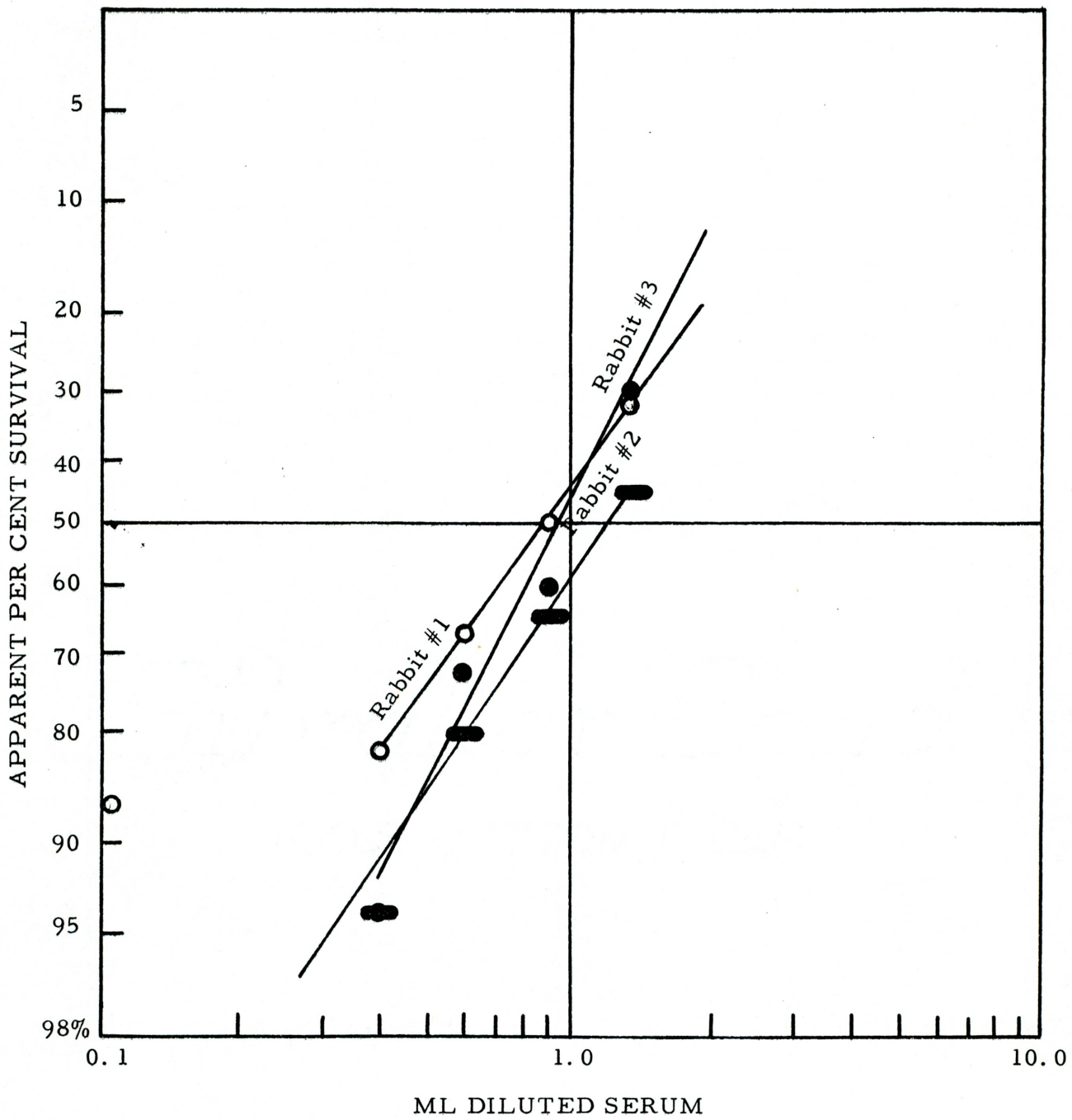


Table 8

BACTERICIDAL TITERS OF RABBIT SERA AT SIXTH BLEEDING
(Eighteen days after initial injection)

		Rabbit #1 Serum (1:4000)					Rabbit #3 Serum (1:5000)					Comp.	Diluent
Ml diluted serum		1.35	0.9	0.6	0.4	0.1	1.35	0.9	0.6	0.4	0.1	---	---
% Trans. at 0 hours		100	100	100	100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit	91	91	85	80	70	87	93	86	86	70	67	48
	Optical density	.04	.04	.075	.10	.16	.065	.03	.07	.07	.16	.175	.32
	Apparent % surv.	23	23	43	57	91	37	17	40	40	92		
5 hr. incub.	% Transmit.	82	80	74	66	57	79	86	76	74	56	54	34.5
	Optical density	.09	.10	.13	.18	.245	.105	.07	.12	.13	.25	.27	.465
	Apparent % surv.	33	37	48	67	91	39	26	44	48	93		
Aver. appt. % surv.		28	30	46	62	91	38	22	42	44	92		
50% surv. end point		0.54 ml of a 1:4000 dilution					0.42 ml of a 1:5000 dilution						
Serum titer*		7407					11,905						

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 56.5% survival

Table 8

BACTERICIDAL TITERS OF RABBIT SERA AT SIXTH BLEEDING
(Eighteen days after initial injection)

		Rabbit #2 Serum (1:40,000)					Comp.	Diluent
Ml diluted serum		1.35	0.9	0.6	0.4	0.1	---	---
% Trans. at 0 hours		100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	91	86	80	75	70	70.5	63.5
	Optical density	.04	.07	.10	.125	.16	.155	.198
	Apparent % Surv.	26	45	65	81	100		
5 hr. incub.	% Transmit.	85	76	68	63	59	59.5	51.5
	Optical density	.075	.12	.170	.20	.23	.225	.292
	Apparent % surv.	33	53	76	89	100		
Aver. appt. % surv.		30	49	70	85	100		
50% surv. end point		0.90 ml of a 1:40,000 dilution						
Serum titer*		44,444						

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 56.5% survival

GRAPH 8

BACTERICIDAL TITERS AT SIXTH BLEEDING

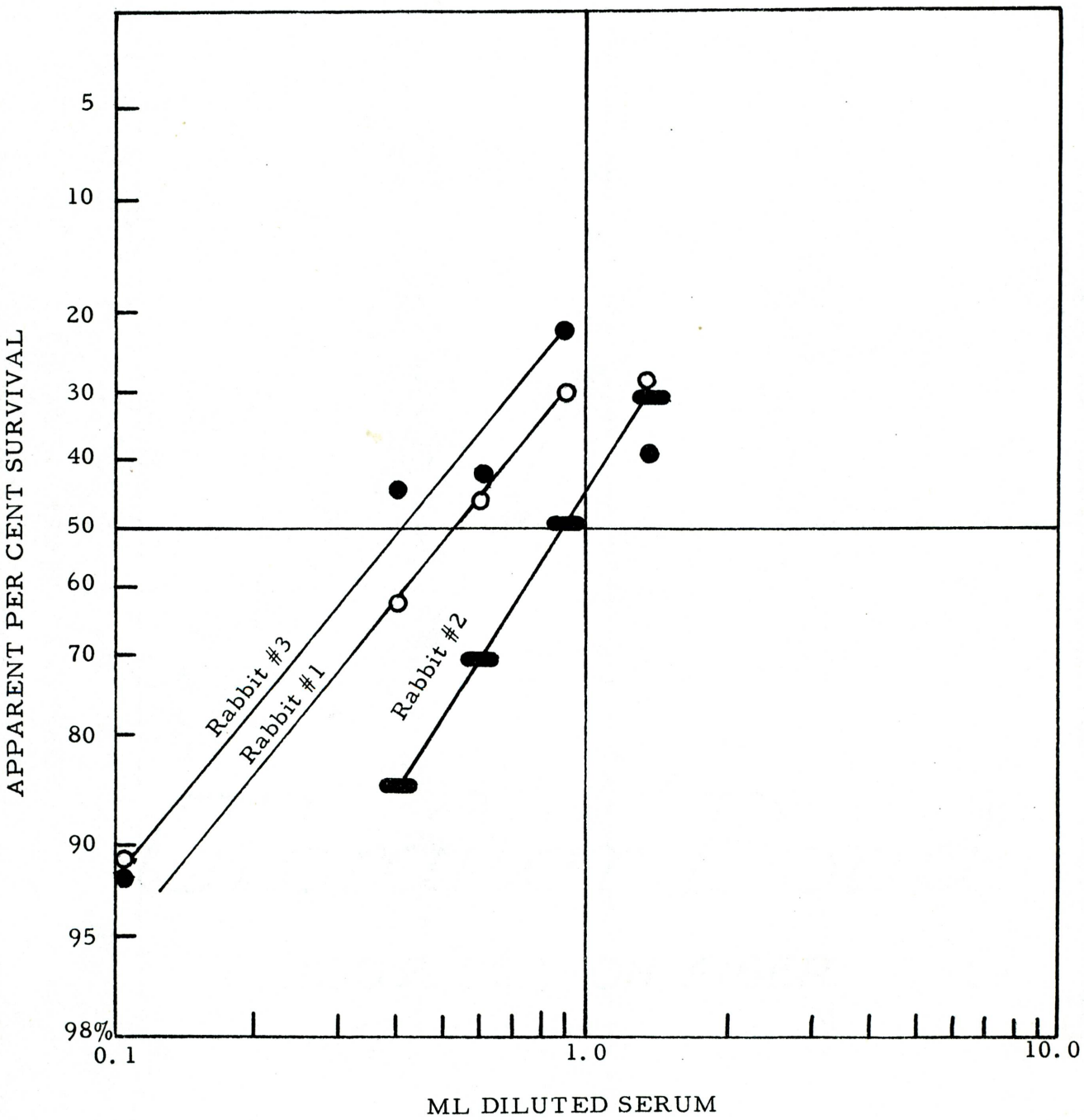


Table 9

BACTERICIDAL TITERS OF RABBIT SERA AT SEVENTH BLEEDING
(Twenty-three days after initial injection)

		Rabbit #1 Serum (1:1000)					Rabbit #2 Serum (1:5000)					Comp.	Diluent
Ml diluted serum		1.35	0.9	0.6	0.4	0.1	1.35	0.9	0.6	0.4	0.1	---	---
% Trans. at 0 hour		100	100	100	100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	90	89	87	79	64	86	90	89	85	65	60.5	51.5
	Optical density	.05	.055	.065	.105	.195	.07	.05	.055	.075	.19	.218	.292
	Apparent % surv.	23	25	30	48	89	32	23	25	34	87		
5 hr. incub.	% Transmit.	88	88	83	75	60	84	88	87	83	60	53	43.5
	Optical density	.06	.06	.085	.125	.22	.08	.06	.065	.085	.22	.28	.365
	Apparent % surv.	21	21	30	45	79	29	21	23	30	79		
Aver. appt. % surv.		22	23	30	46	84	30	22	24	32	83		
50% surv. end point		0.36 ml of a 1:1000 dilution					0.25 ml of a 1:5000 dilution						
Serum titer*		2778					20,000						

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 76% survival

Table 9

BACTERICIDAL TITER OF RABBIT SERUM AT SEVENTH BLEEDING
(Twenty-three days after initial injection)

		Rabbit #3 Serum (1:4000)				Comp.	Diluent
Ml diluted serum		1.35	0.9	0.6	0.3	---	---
% Trans. at 0 hour		100	100	100	100	100	100
4 hr. incub.	% Transmit.	92	92	90	78	66	56.5
	Optical density	.035	.035	.05	.11	.18	.248
	Apparent % surv.	19	19	28	61		
5 hr. incub.	% Transmit	88	87	83	65	53	42
	Optical density	.06	.065	.085	.19	.28	.38
	Apparent % surv.	21	23	30	68		
Aver. appt. % surv.		20	21	29	64		
50% surv. end point		0.43 ml of a 1:4000 dilution					
Serum titer*		9302					

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 76% survival

GRAPH 9

BACTERICIDAL TITERS AT SEVENTH BLEEDING

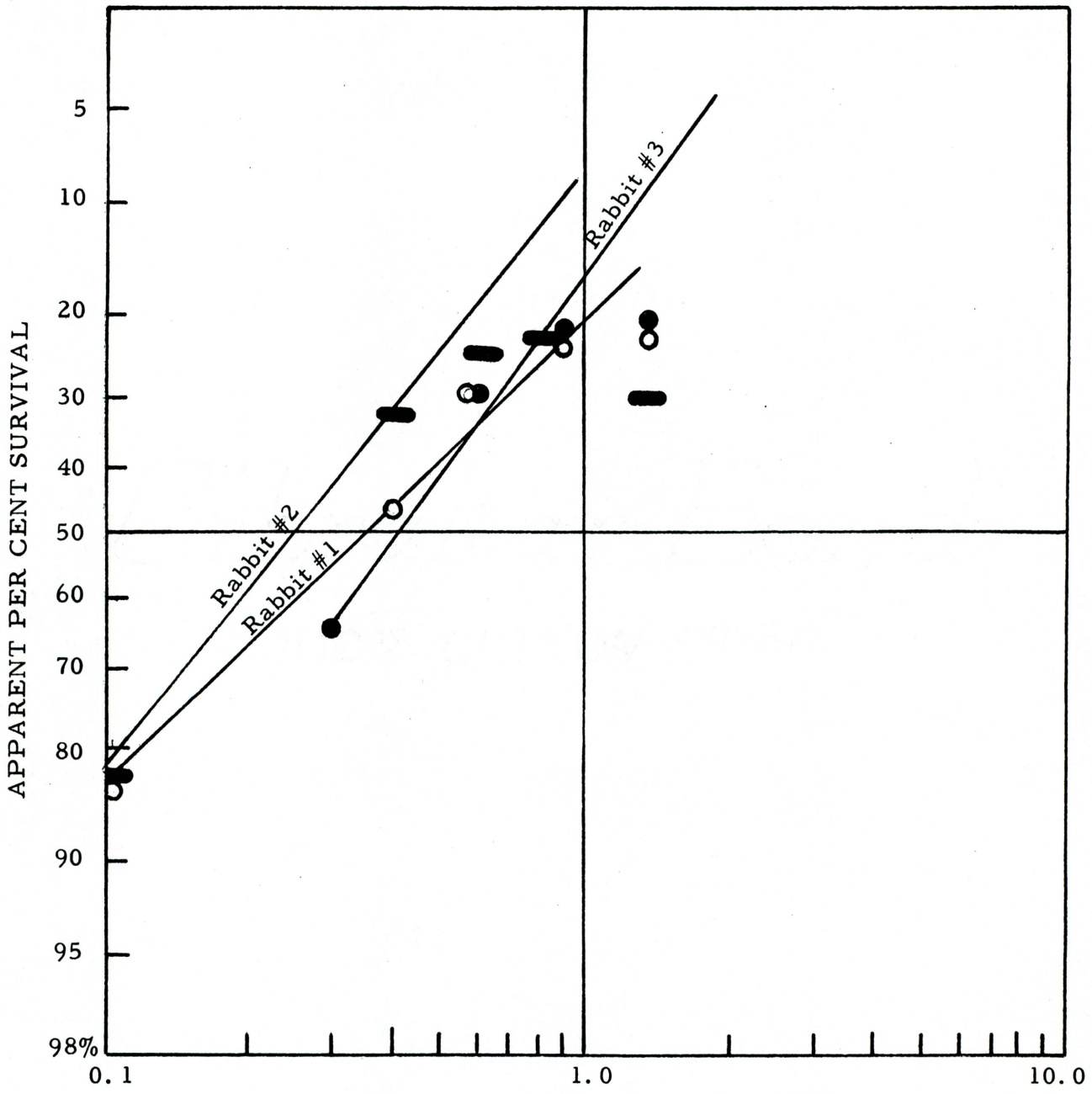


Table 10

BACTERICIDAL TITERS OF RABBIT SERA AT EIGHTH BLEEDING
(Twenty-nine days after initial injection)

		Rabbit #1 Serum (1:1000)						Comp.	Dil.	Rabbit #3 Serum (1:1000)						Comp.	Dil.
Ml diluted serum		2.7	1.35	0.9	0.6	0.4	0.1	---	---	2.7	1.35	0.9	0.6	0.4	0.1	---	--
% Trans. at 0 hours		100	100	100	100	100	100			100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	94	94	94	93	88	73	71	60	93	93	96	96	96	80	72.5	61.5
	Optical density	.025	.025	.025	.03	.06	.135	.15	.22	.03	.03	.017	.017	.017	.10	.138	.212
	Apparent % surv.	17	17	17	20	40	90			22	22	12	12	12	72		
5 hr. incub.	% Transmit.	89	90	91	89	81	63	59.5	47.5	89	90	92	92	91	69	60.5	48.5
	Optical density	.055	.05	.04	.055	.095	.20	.225	.325	.055	.05	.035	.035	.04	.165	.218	.315
	Apparent % surv.	24	22	18	24	42	89			25	23	16	16	18	76		
Aver. appt. % surv.		20	20	18	22	41	90			24	22	14	14	15	74		
50% surv. end point		0.32 ml of a 1:1000 dil.								0.17 ml of a 1:1000 dil.							
Serum titer*		3125								5882							

Comp. 68.5% surv. Comp. 67% surv.

*Dilution of serum at which 1.0 ml produces 50% survival end point

Table 10

BACTERICIDAL TITERS OF RABBIT SERA AT EIGHTH BLEEDING
(Twenty-nine days after initial injection)

		Rabbit #2** Serum (1:20,000)					Comp.	Diluent
Ml diluted serum		2.7	1.35	0.9	0.6	0.4	---	---
% Trans. at 0 hours		100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	94	89	85	75	73	68	58
	Optical density	.025	.055	.075	.125	.135	.17	.24
	Apparent % surv.	15	32	44	74	79		
5 hr. incub.	% Transmit.	90	82	76	65	62	59	47
	Optical density	.05	.09	.12	.19	.21	.23	.33
	Apparent % surv.	22	39	52	83	91		
Aver. appt. % surv.		18	36	48	78	85		
50% surv. end point		1.0 ml of a 1:20,000 dilution						
Serum titer*		20,000						

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 67% survival

**Rabbit died from trauma of cardiac puncture

GRAPH 10

BACTERICIDAL TITERS AT EIGHTH BLEEDING

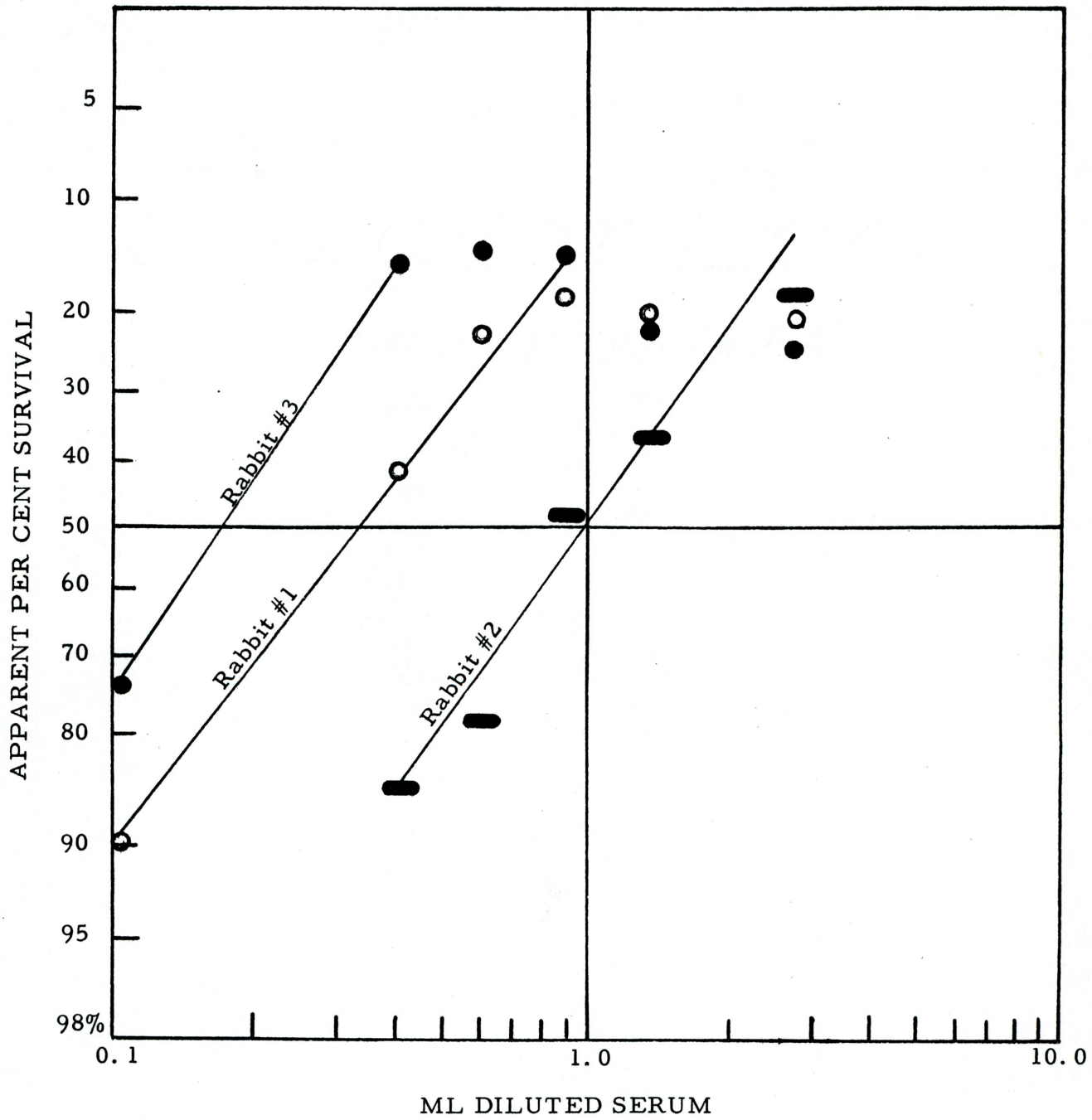


Table 11

BACTERICIDAL TITERS OF RABBIT SERA AT NINTH BLEEDING
(Thirty-three days after initial injection)

		Rabbit #1 Serum (1:2000)				Comp.	Dil.	Rabbit #3 Serum (1:4000)				Comp.	Dil.
Ml diluted serum		1.35	0.9	0.6	0.3	---	---	1.35	0.9	0.6	0.3	---	---
% Trans. at 0 hours		100	100	100	100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	93	89	83	76	68.5	57	92	88	80	73	66	56.5
	Optical density	.03	.055	.085	.12	.168	.245	.035	.06	.10	.135	.18	.248
	Apparent % surv.	18	33	51	71			19	33	56	75		
5 hr. incub.	% Transmit.	90	84	74	64	55.5	42	88	80	68	62	53	42
	Optical density	.05	.08	.13	.195	.255	.38	.06	.10	.17	.21	.28	.38
	Apparent % surv.	20	31	51	77			21	36	61	75		
Aver. appt. % surv.		19	32	51	74			20	34	58	75		
50% surv. end point		0.58 ml of a 1:2000 dil.						0.60 ml of a 1:4000 dil.					
Serum titer*		3448						6667					
		Comp. 68% surv.						Comp. 73.5% surv.					

*Dilution of serum at which 1.0 ml produces 50% survival end point

GRAPH 11

BACTERICIDAL TITERS AT NINTH BLEEDING

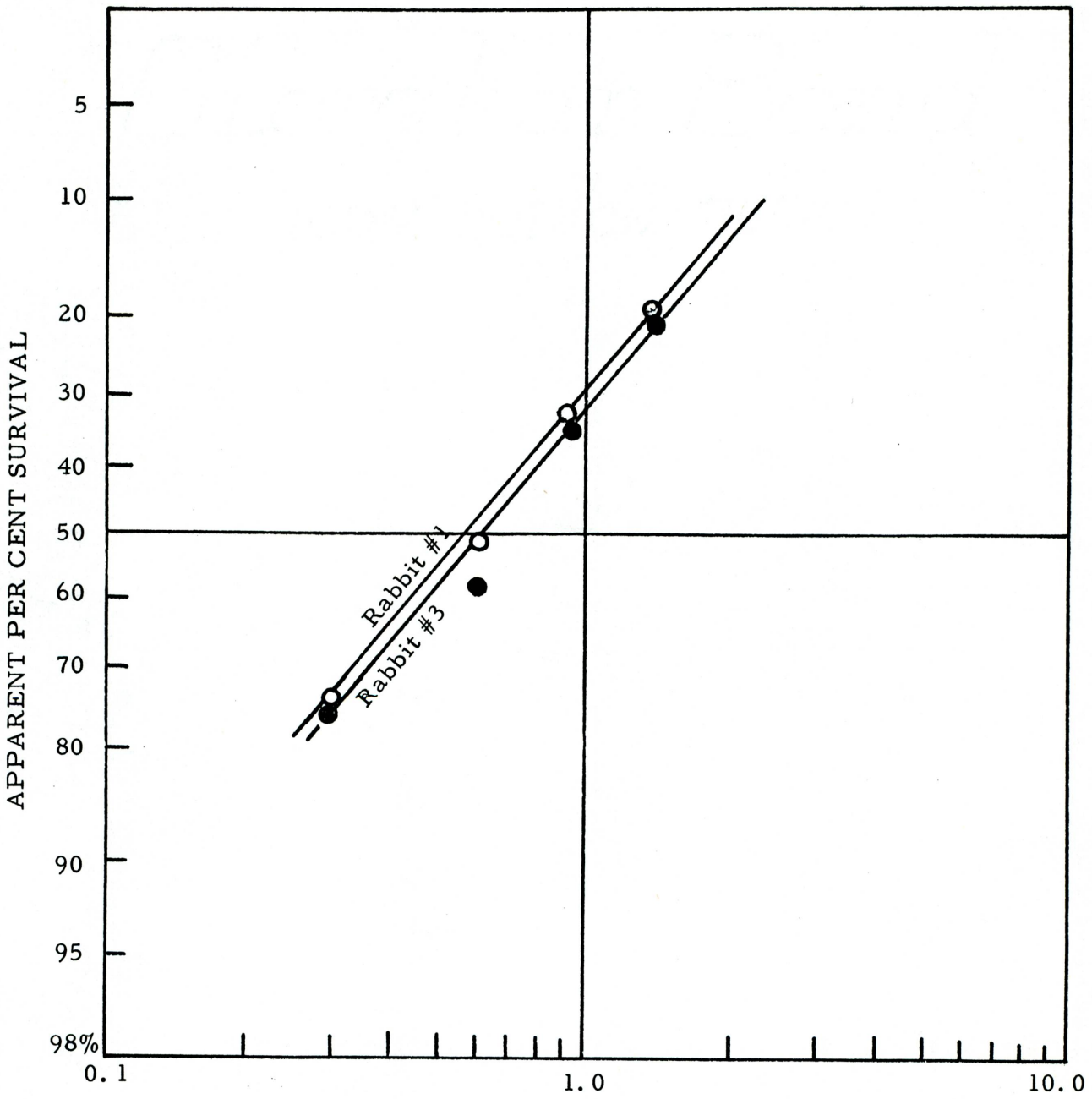


Table 12

BACTERICIDAL TITERS OF RABBIT SERA AT TENTH BLEEDING
(Thirty-eight days after initial injection)

		Rabbit #1 Serum (1:2000)				Comp.	Diluent	Rabbit #3 Serum (1:2000)				Comp.	Diluent
Ml diluted serum		1.35	0.9	0.6	0.3	---	---	1.35	0.9	0.6	0.3	---	---
% Trans. at 0 hours		100	100	100	100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	87	80	77	74	68.5	57	94	94	90	76	66	56.5
	Optical density	.065	.10	.115	.13	.168	.245	.025	.025	.05	.12	.18	.248
	Apparent % surv.	39	59	68	77			14	14	28	67		
5 hr. incub.	% Transmit.	80	68	65	62	55.5	42	91	90	83	65	53	42
	Optical density	.10	.17	.19	.21	.255	.38	.04	.05	.085	.19	.28	.38
	Apparent % surv.	39	67	75	82			14	18	30	68		
Aver. appt. % surv.		39	63	72	80			14	16	29	68		
50% surv. end point		1.05 ml of a 1:2000 dil.						0.43 ml of a 1:2000 dil.					
Serum titer*		1905						4651					
		Comp. 68% surv.						Comp. 73.5% surv.					

*Dilution of serum at which 1.0 ml produces 50% survival end point

GRAPH 12

BACTERICIDAL TITERS AT TENTH BLEEDING

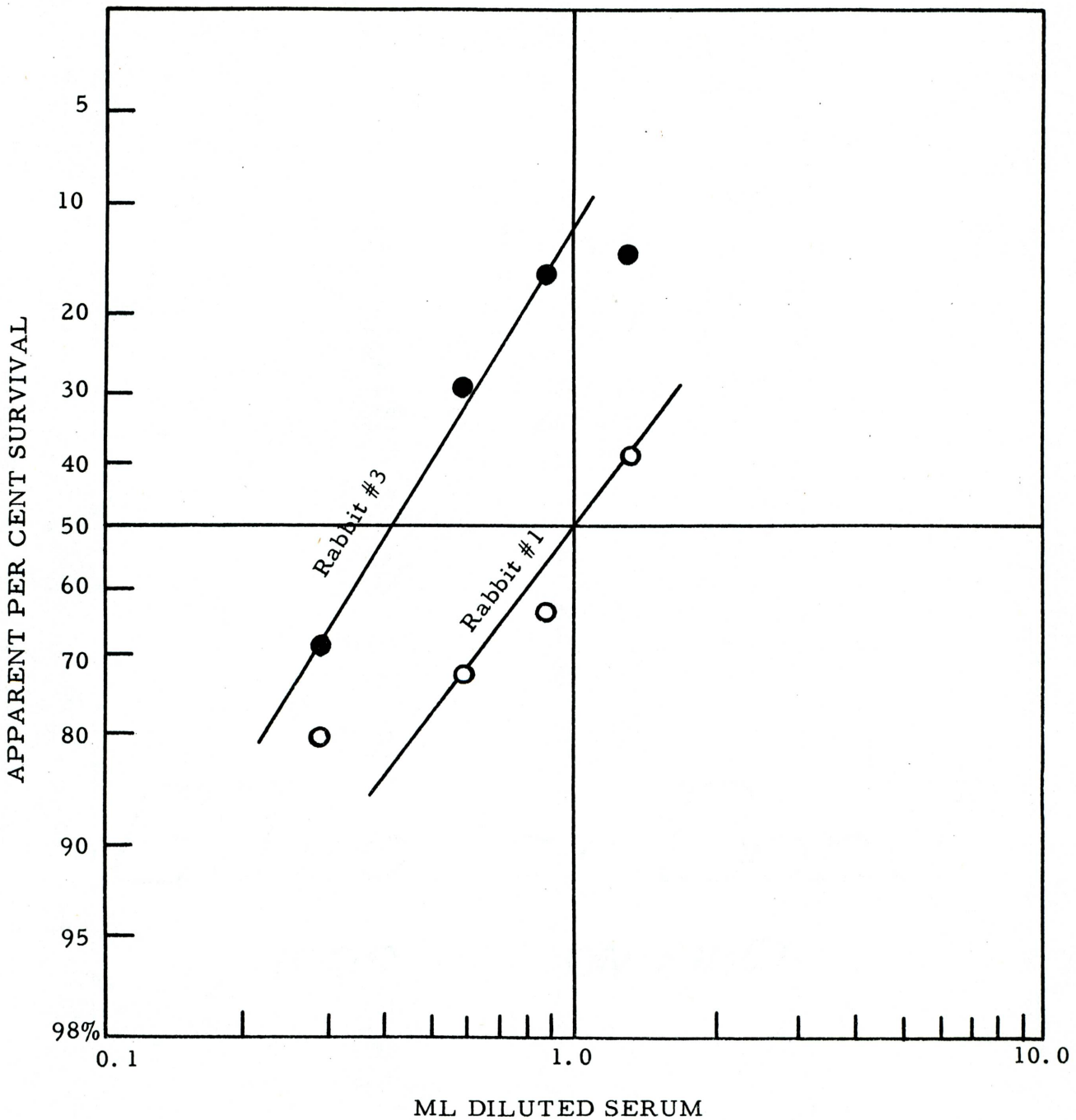


Table 13

BACTERICIDAL TITERS OF RABBIT SERA AT ELEVENTH BLEEDING
(Forty-five days after initial injection)

		Rabbit #1 Serum (1:1000)					Comp.	Dil.	Rabbit #3 Serum (1:2000)				Comp.	Dil.
Ml diluted serum		2.7	1.35	0.9	0.6	0.3	---	---	1.35	0.9	0.6	0.3	---	---
% Trans. at 0 hours		100	100	100	100	100	100	100	100	100	100	100	100	100
5 hr. incub. 4 hr. incub.	% Transmit.	89	87	82	74	68	63.5	48.5	93	90	80	72	66	56.5
	Optical density	.055	.065	.09	.13	.17	.198	.315	.03	.05	.10	.14	.18	.248
	Apparent % surv.	28	33	45	66	86			17	28	56	78		
	% Transmit.	82	79	71	63	58	52	37.5	90	81	70	62	53	42
	Optical density	.09	.105	.15	.20	.24	.29	.425	.05	.095	.16	.21	.28	.38
	Apparent % surv.	31	36	52	69	83			18	34	57	75		
Aver. appt. % surv.		30	34	48	68	84			18	31	56	76		
50% surv. end point		0.86 ml of a 1:1000 dil.							0.57 ml of a 1:2000 dil.					
Serum titer*		1163							3509					
		Comp. 67.5% surv.							Comp. 73.5% surv.					

*Dilution of serum at which 1.0 ml produces 50% survival end point

GRAPH 13

BACTERICIDAL TITERS AT ELEVENTH BLEEDING

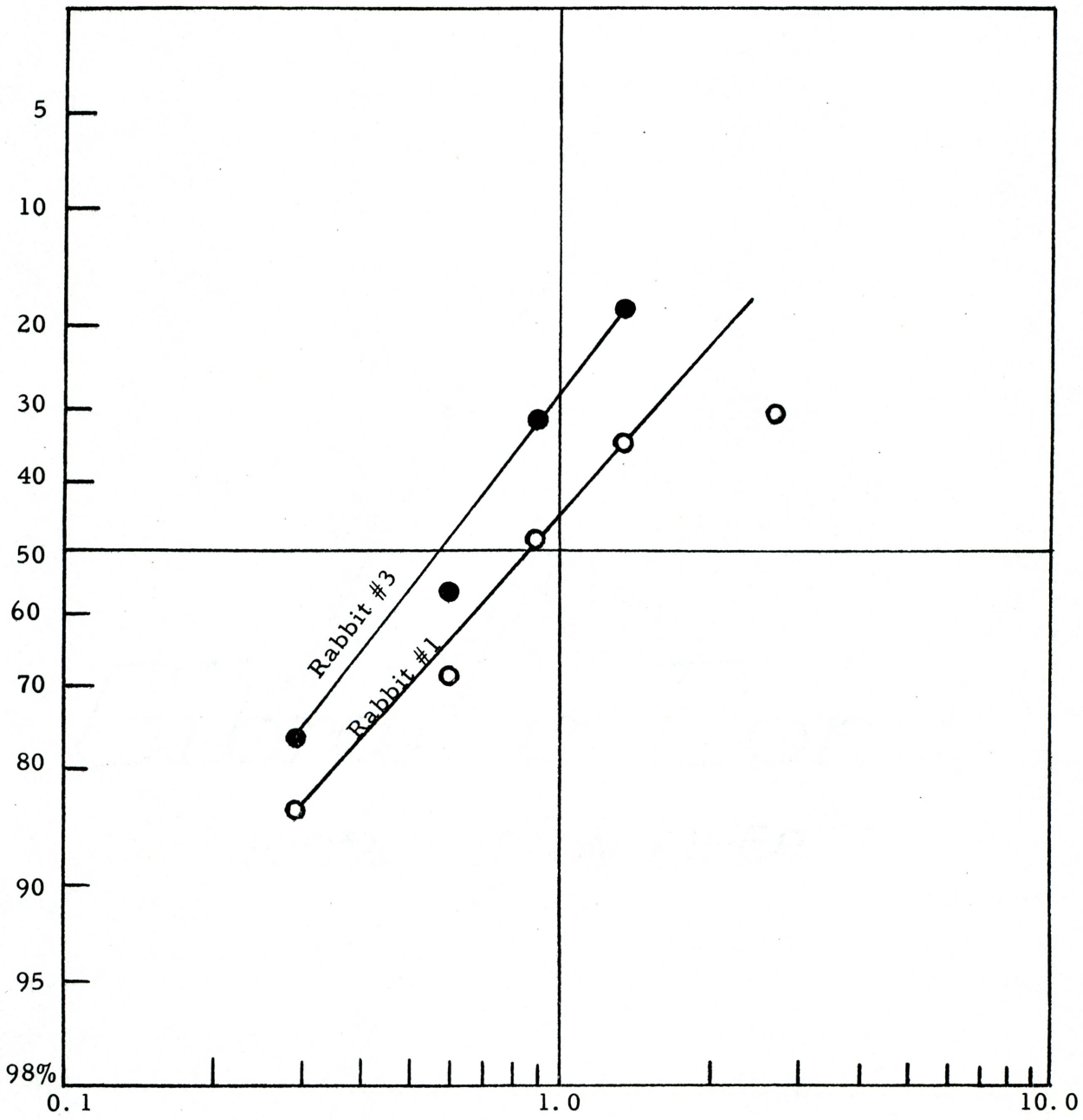


Table 14

BACTERICIDAL TITERS OF RABBIT SERA AT TWELFTH BLEEDING
(Fifty-nine days after initial injection)

		Rabbit #1 Serum (1:1000)						Comp.	Dil.	Rabbit #3 Serum (1:2000)						Comp.	Dil.
Ml diluted serum		2.7	1.35	1.2	0.9	0.6	0.3	---	---	2.7	1.35	1.2	0.9	0.6	0.4	---	---
% Trans. at 0 hours		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	90	84	81	76	71	68	63.5	48.5	84	79	75	71	65	64	61.5	48
	Optical density	.05	.08	.095	.12	.15	.17	.198	.315	.08	.105	.125	.15	.19	.195	.212	.32
	Apparent % surv.	25	40	48	61	76	86			38	50	59	71	90	92		
5 hr. incub.	% Transmit	84	74	72	65	61	59	52	37.5	77	69	64	63	55	54	50.5	37.5
	Optical density	.08	.13	.14	.19	.215	.23	.29	.435	.115	.165	.195	.20	.26	.27	.298	.425
	Apparent % surv.	28	45	48	66	74	79			39	55	65	67	87	91		
Aver. appt. % surv.		26	42	48	64	75	82										
50% surv. end point		1.12 ml of a 1:1000 dilution								1.40 ml of a 1:2000 dilution							
Serum titer*		893								1429							
		Comp. 65% surv.								Comp. 68% surv.							

*Dilution of serum at which 1.0 ml produces 50% survival end point

GRAPH 14

BACTERICIDAL TITERS AT TWELFTH BLEEDING

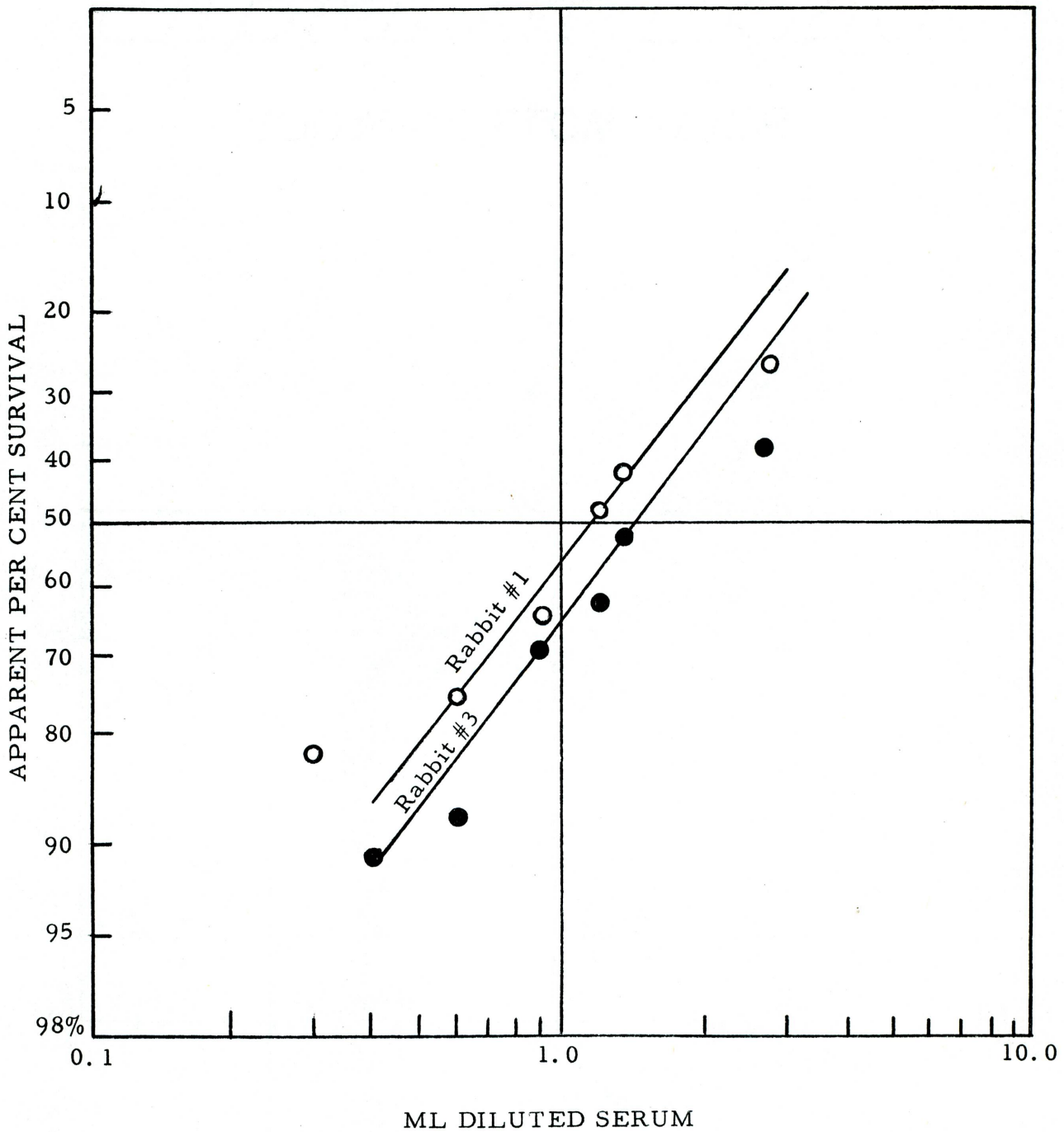


Table 15

BACTERICIDAL TITERS OF RABBIT SERA AT THIRTEENTH BLEEDING
(Sixty-six days after initial injection)

		Rabbit #1 Serum (1:300)					Comp.	Dil.	Rabbit #3 Serum (1:300)					Comp.	Dil.
Ml diluted serum		4.05	1.35	0.9	0.6	0.15	---	---	1.35	0.9	0.6	0.2	0.1	---	---
% Trans. at 0 hours		100	100	100	100	100	100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	94	94	92	88	74	70.5	66	95	94	91	77	73	64.5	60.5
	Optical density	.025	.025	.035	.06	.13	.155	.18	.02	.025	.04	.115	.135	.192	.218
	Apparent % surv.	16	16	23	39	84			10	13	21	60	70		
5 hr. incub.	% Transmit.	87	86	82	78	60	55	49	88	86	82	61	56	49.5	43
	Optical density	.065	.07	.09	.11	.22	.26	.31	.06	.07	.09	.215	.25	.305	.37
	Apparent % surv.	25	27	35	42	85			20	23	29	71	82		
Aver. appt. % surv.		20	22	29	40	84			15	18	25	66	76		
50% surv. end point		0.40 ml of a 1:300 dil.							0.31 ml of a 1:300 dil.						
Serum titer*		750							968						
		Comp. 85% surv.							Comp. 85% surv.						

*Dilution of serum at which 1.0 ml produces 50% survival end point

GRAPH 15

BACTERICIDAL TITERS AT THIRTEENTH BLEEDING

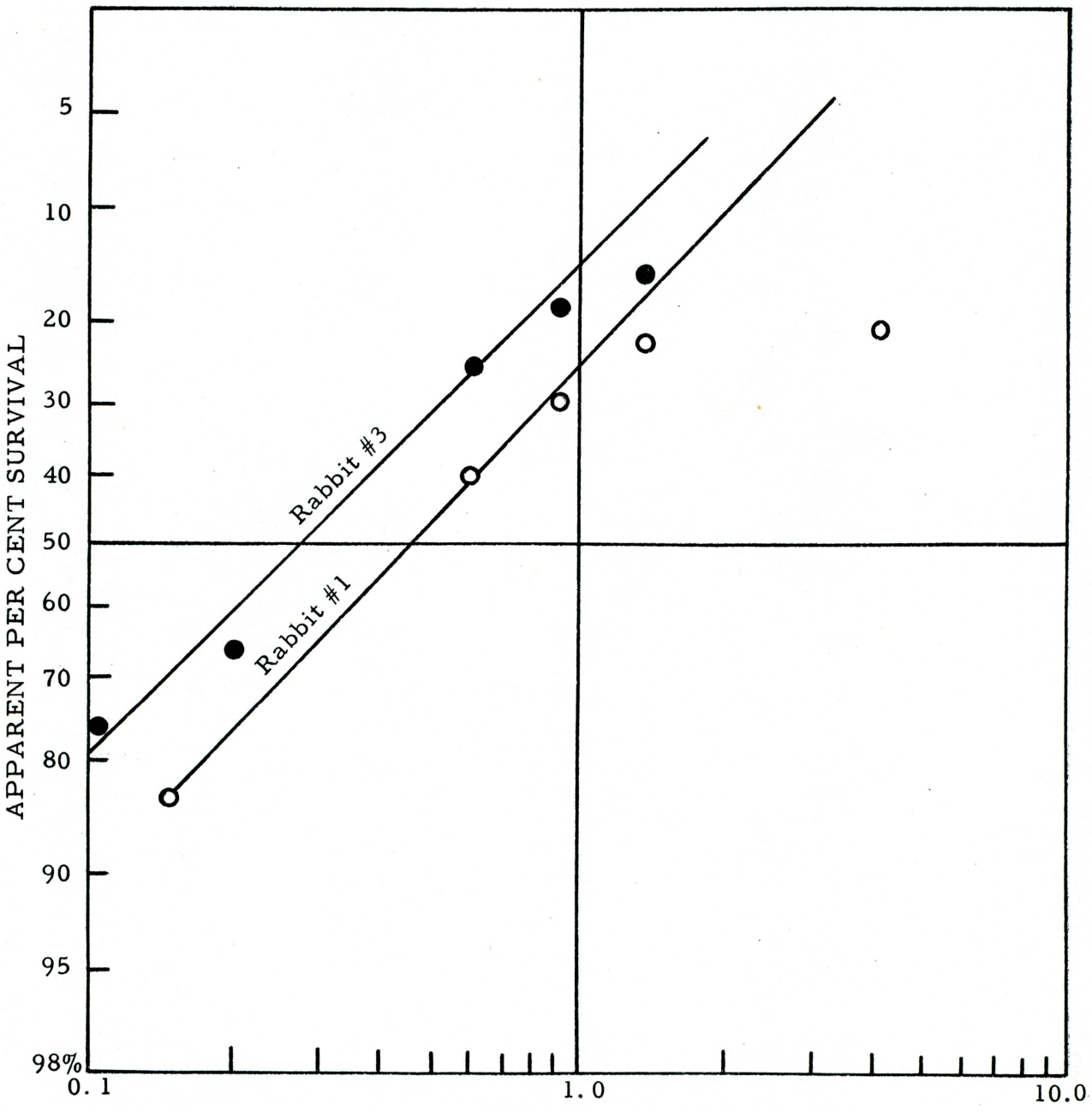


Table 16

BACTERICIDAL TITERS OF RABBIT SERA AT FOURTEENTH BLEEDING
(Seventy-five days after initial injection)

		Rabbit #1 Serum (1:300)					Comp.	Dil.	Rabbit #3** Serum (1:100)						Comp.	Dil.
Ml diluted serum		4.05	1.35	0.9	0.6	0.15	---	---	1.35	0.9	0.6	0.3	0.2	0.1	---	---
% Trans. at 0 hours		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	84	90	82	78	71	70.5	66	95	92	86	78	73	70	64.5	60.5
	Optical density	.025	.05	.09	.11	.15	.155	.18	.02	.035	.07	.11	.135	.16	.192	.218
	Apparent % surv.	16	32	58	71	97			10	18	36	57	70	83		
5 hr. incub.	% Transmit.	87	81	71	65	59	55	49	87	83	73	60	57	52	42.5	39.5
	Optical density	.065	.095	.15	.19	.23	.26	.31	.065	.09	.135	.22	.245	.29	.375	.405
	Apparent % surv.	25	36	58	73	98			17	24	36	59	65	78		
Aver. appt. % surv.		20	34	58	72	98			14	21	36	58	68	80		
50% surv. end point		0.98 ml of a 1:300 dilution							0.36 ml of a 1:100 dilution							
Serum titer*		306							278							
		Comp. 85% surv.							Comp. 90.5% surv.							

*Dilution of serum at which 1.0 ml produces 50% survival end point

**Rabbit died after giving birth.

GRAPH 16

BACTERICIDAL TITERS AT FOURTEENTH BLEEDING

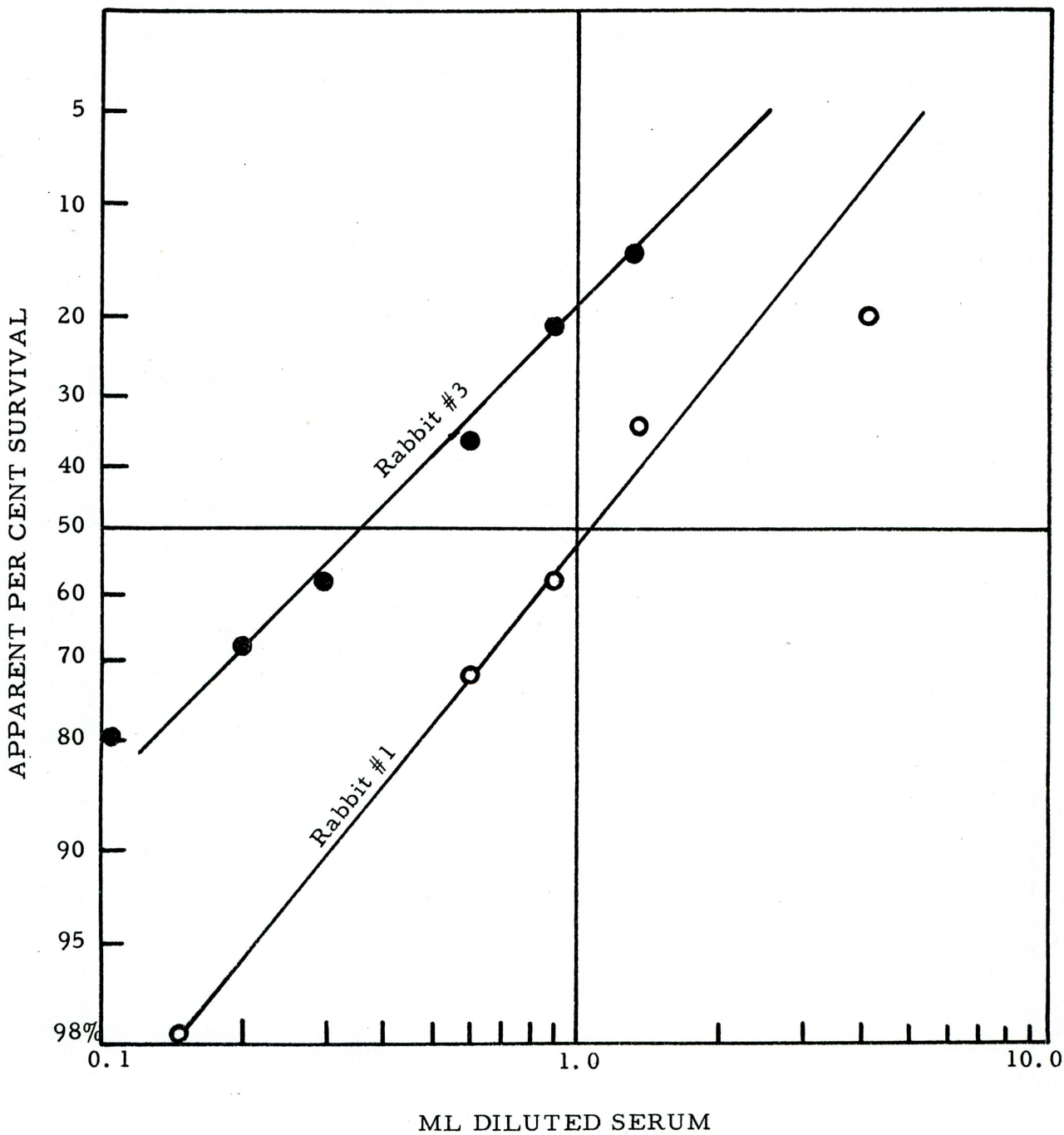


Table 17

BACTERICIDAL TITER OF RABBIT SERUM AT FIFTEENTH BLEEDING
(Eighty-six days after initial injection)

		Rabbit #1 Serum (1:300)					Comp.	Diluent
Ml diluted serum		4.05	1.35	0.9	0.6	0.15	---	---
% Trans. at 0 hours		100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	91	83	79	76	68	68.5	65
	Optical density	.04	.085	.105	.12	.17	.168	.19
	Apparent % surv.	24	51	62	72	100		
5 hr. incub.	% Transmit.	87	74	66	63	58	55	49
	Optical density	.065	.13	.18	.20	.24	.26	.31
	Apparent % surv.	25	50	69	77	92		
Aver. appt. % surv.		24	50	66	74	96		
50% surv. end point		1.35 ml of a 1:300 dilution						
Serum titer*		222						

*Dilution of serum at which 1.0 ml produces 50% survival end point

Complement 86% surv.

GRAPH 17

BACTERICIDAL TITERS AT FIFTEENTH BLEEDING

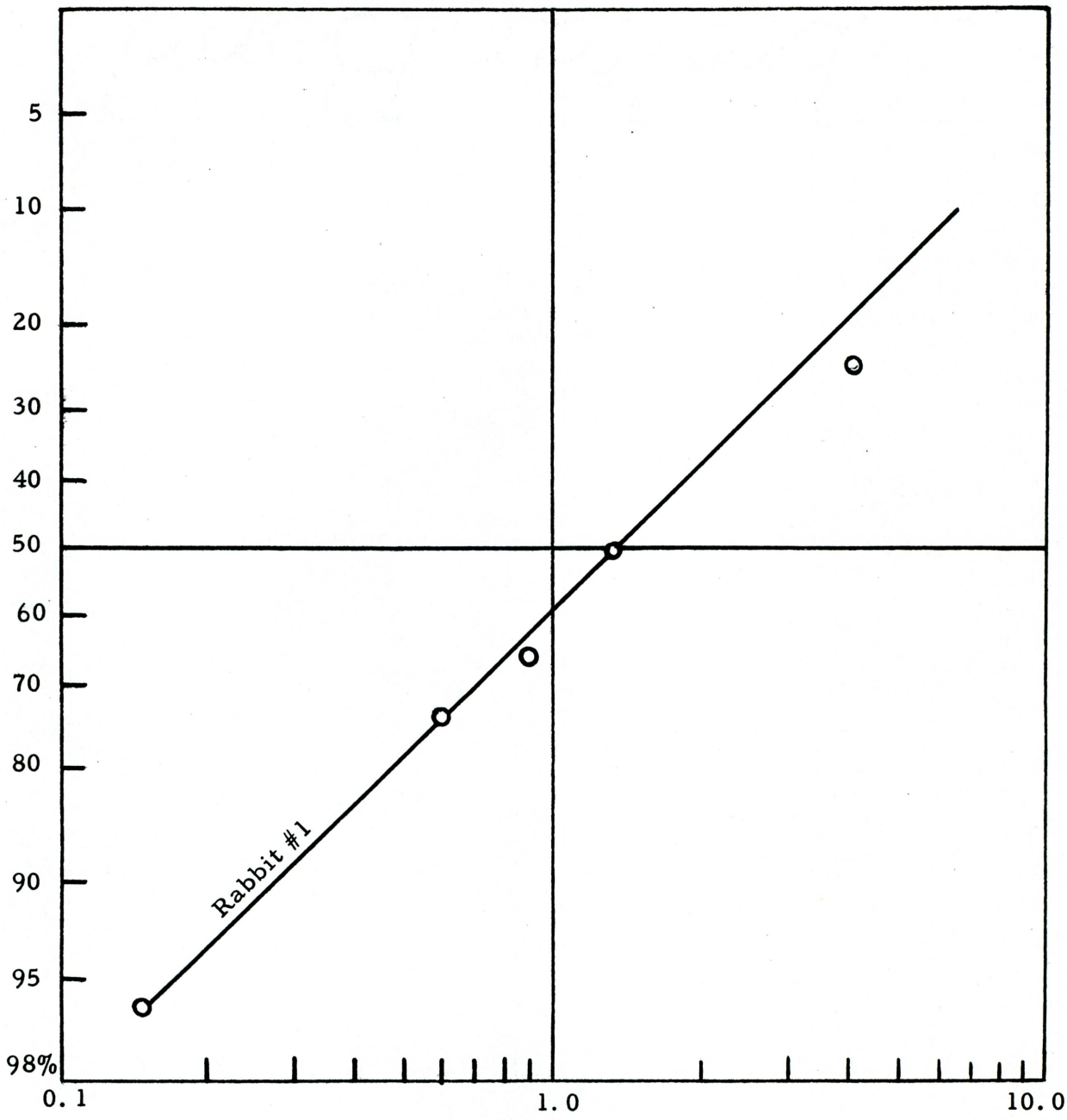


Table 18

BACTERICIDAL TITER OF RABBIT SERUM AT SIXTEENTH BLEEDING
(Eighty-eight days after initial injection)

		Rabbit #1 Serum (1:100)				Comp.	Diluent
Ml diluted serum		1.35	0.9	0.6	0.3	---	---
% Trans. at 0 hours		100	100	100	100	100	100
4 hr. incub.	% Transmit.	92	89	84	80	75.5	68
	Optical density	.035	.055	.08	.10	.122	.17
	Apparent % surv.	29	45	66	82		
5 hr. incub.	% Transmit.	85	80	75	69	63	53
	Optical density	.075	.10	.126	.165	.20	.28
	Apparent % surv.	38	50	63	83		
Aver. appt. % surv.		34	48	64	82		
50% surv. end point		.86 ml of a 1:100 dilution					
Serum titer*		116					

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 71.5% survival

GRAPH 18

BACTERICIDAL TITERS AT SIXTEENTH BLEEDING

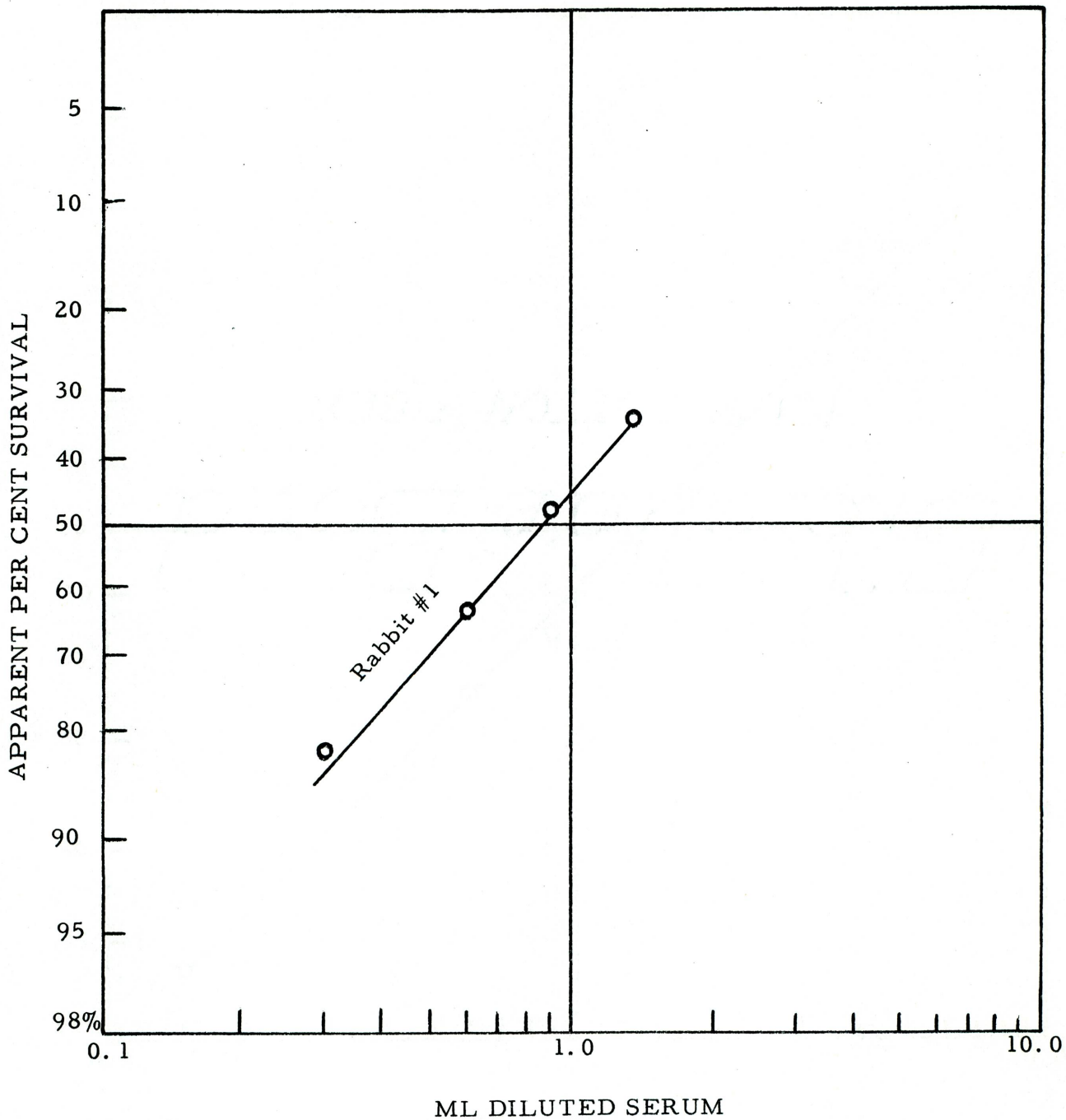


Table 19

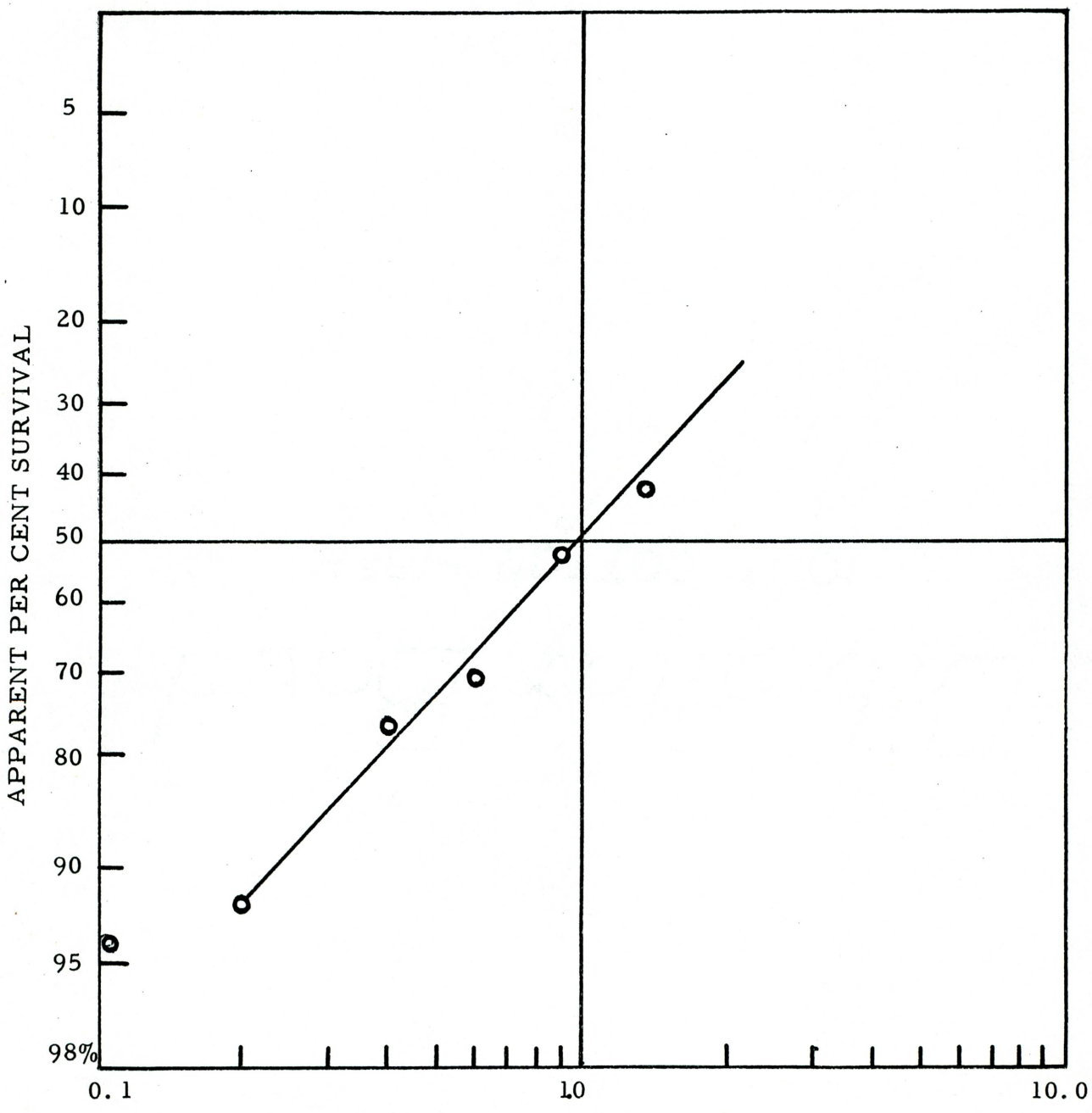
BACTERICIDAL TITER OF RABBIT SERUM AT SEVENTEENTH BLEEDING
(Ninety-two days after initial injection)

		Rabbit #1 Serum (1:6000)							Comp.	Diluent
Ml diluted serum		16.2	1.35	0.9	0.6	0.4	0.2	0.1	---	---
% Trans at 0 hours		100	100	100	100	100	100	100	100	100
4 hr. incub.	% Transmit.	95	90	88	84	82	78	78	76.5	64.5
	Optical density	.02	.05	.06	.08	.09	.11	.11	.118	.192
	Apparent % surv.	17	42	51	68	76	93	93		
5 hr incub.	% Transmit.	89	83	79	72	71	66	65	63.5	48.5
	Optical density	.055	.085	.105	.14	.15	.18	.19	.198	.315
	Apparent % surv.	28	43	53	71	76	91	96		
Aver. appt. % surv.		22	42	52	70	76	92	94		
50% surv. end point		0.97 ml of a 1:6000 dilution								
Serum titer*		6186								

*Dilution of serum at which 1.0 ml produces 50% survival end point Complement 63% survival

GRAPH 19

BACTERICIDAL TITERS AT SEVENTEENTH BLEEDING



Serology of Pre-immunization Sera

The first bleeding was performed for the purpose of establishing the pre-immunization bactericidal and agglutinating titers of the three rabbits. As predicted, Salmonella pullorum bactericidal antibody was present in low concentrations naturally in the sera of the young rabbits. All three bactericidal titers were 10 or less in value but greater than zero (3.1, 9.6, and 2.0 for rabbit #1, rabbit #2 and rabbit #3 respectively). See table and graph 3 for the experimental data. Rabbit #2, the oldest of the three had the highest titer of natural bactericidal antibody probably produced in response to greater antigenic stimulation in its environment. The agglutination tests were negative for all three rabbits before immunization.

Thus, at the onset of the study, the normal sera from three young rabbits demonstrated the earlier appearance of bactericidal antibodies and/or the greater sensitivity of the bactericidal test as compared to agglutination.

Bactericidal Determination Example

Data from the fourth bleeding serve as a good example to explain the bactericidal determination technique. This blood was drawn from the three rabbits ten days after the first immunization. See table and graph 6. These results represent the maximum immune bactericidal antibody responses of both rabbits #1 and #2. The bactericidal antibody

level of rabbit #3 was increasing but had not yet reached its maximum concentration.

Each serum was diluted to a concentration, suggested by preliminary testing, such that different amounts of that dilution would produced apparent per cent kills on both sides of the 50% level. The three serum samples were diluted 1:500, 1:30,000, and 1:5,000 respectively for rabbits #1, #2, and #3 such that the apparent per cent survivals plotted against the log of the amount of diluted serum contained in each tube produced three straight lines on probit graph paper which crossed the 50% survival level for each rabbit giving titers of 17,540, 51,720, and 12,500.

A typical "prozone" or Neisser-Wechsberg effect can be seen in the titer determinations of rabbits #1 and #3 where the maximum 1.35 ml quantities of diluted serum demonstrated less killing than the smaller amounts. Smaller amounts of serum produced a typical straight line response while the apparent per cent survivals of the 1.35 ml amounts fell far off to the right indicating a decrease in killing action.

The most accepted explanation of this phenomenon is that it similates the common prozone effect occurring in agglutination tests. There is an optimum antigen antibody ratio for agglutination to occur. In this range, antibody combines with antigen and forms a bridge for

uniting more antigen particles such that visible aggregates are formed. When this antibody level is exceeded to the point that it completely coats the antigen there are no more points of contact for antigen antibody linkage to occur and agglutination is inhibited. A similar antigen antibody ratio occurs for sensitization of the bacteria to the killing action of complement. When this is exceeded a decrease in killing efficiency occurs up to a complete cessation. Since antigen alone attracts complement weakly and antibody alone not at all it may be that the complement is not attracted. In some cases it may occur that although the complement combines with the antigen antibody complex unit, the antibody completely coating the surface of the live bacteria eliminates points of contact necessary for lysis or killing to occur.

Maximum response slopes occur for rabbits #1 and #2 and will be discussed in the section on response slopes.

The complement control tubes used exhibited an apparent per cent survival of 74.5% as compared to the diluent control tubes.

Bactericidal Antibody Response

As indicated by the data in Table 2 and Graphs 1 and 2ABC, all three rabbits responded to immunization after an initial lag of two to five days, and by the third bleeding (two days after the second

immunization) a sharp rise in antibody production had occurred. The fourth and final immunization dose appeared to cause a slight decrease in antibody titer for rabbits #1 and #2. A less concentrated antigen dose might have produced a more beneficial response as it is thought that the absorption of antibody by the large amount of antigen may remove available antibody.

Maximum bactericidal antibody titers of 17,540 and 51,720 were attained by rabbits #1 and #2 at the fourth bleeding (ten days after the first immunization dose and two days after the third). Rabbit #3 responded a little slower reaching a maximum antibody concentration of 15,960 at the fifth bleeding (fourteen days after the initial immunization dose and two days after the fourth).

None of the maximum peak bactericidal antibody titers were maintained for a long duration of time. Rabbit #2 retained the highest concentration of bactericidal antibody and this lasted for only four days before a significant drop occurred. Rabbits #1 and #3 exhibited a sharp drop in concentration within four days of reaching their maximum titers.

It was possible to observe the residual bactericidal antibody titers at regular time intervals in rabbits #1 and #3 for a period of almost three months. Unfortunately, rabbit #2 died from trauma following a cardiac puncture at the eighth bleeding. This was most unfortunate

since its response to immunization was the greatest of the three rabbits and for the first month of immunization exhibited the least drop in titer. At the time of its death (twenty-four days after the first immunization), its antibody titer of 20,000 was still greater than the maximum antibody titers of the two younger rabbits.

Rabbits #1 and #3 showed a steady decline in antibody titer after the fourth and fifth bleedings and nineteen days after the fourth immunization had antibody titers of 3,450 and 6,670 respectively. After fifty-three days the significant but weaker titers of 640 and 1,110 remained. Rabbit #1 after seventy-three days had an antibody titer of 220 and immediately after this bleeding was given a booster dose of antigen. The antibody titer jumped to 6,190 six days after the booster dose. No further testing was conducted.

Agglutinin Response

Agglutination titers are given for all three rabbits on Table 2 along with the bactericidal antibody-agglutinin titer ratios. Graphs 2ABC contain the antibody titers (bactericidal and agglutinin) of each individual rabbit for the purpose of demonstrating the sensitivities of the two test methods in their proper perspectives. Graph 2A represents the titers of rabbit #1. Graph 2B represents the titers of rabbit #2. Graph 2C represents the titers of rabbit #3.

Maximum agglutinin production by the three rabbits varied slightly with respect to time, although the sera of all three rabbits had positive agglutinin tests at the third bleeding (see Table 2). Rabbit #3 attained its maximum agglutinin titer of 1,024 ten days after the first immunization at the fourth bleeding. Rabbits #1 and #2 responded a little slower reaching their maximum titers of 256 and 8,192 eighteen and fourteen days after the first immunization at the sixth and fifth bleedings. Rabbit #1 produced a much higher agglutinin titer after the booster dose (1,024) than it had in response to the initial multiple series of four immunizations four days apart.

As predicted, the agglutinin titers were much lower than the corresponding bactericidal titers. Also, there was a greater variation in the agglutinin response between the three rabbits with respect to titer and time of appearance. Rabbit #2 again responded with the highest titer and maintained this level for the longest interval of time, twelve days, even though its bactericidal titer had dropped considerably by this time. All three rabbits exhibited a slower decline in agglutinins than bactericidal antibody. Since the agglutination test is less sensitive than the bactericidal test, the diminished bactericidal titers were still several fold higher than the agglutinin titers (see graphs 2ABC).

Agglutination Versus Bactericidal Action

As evidenced by the data, all three rabbits responded strongly to immunization with Salmonella pullorum and produced high concentrations of both agglutinins and bactericidal antibodies. The bactericidal action of the normal and immune rabbit sera was significantly greater than the agglutinating action. Comparison of the maximum bactericidal and agglutinin titers attained by the three rabbits gives a ratio of 17,540:256 (69:1) for rabbit #1, 51,720:8 (6:1) for rabbit #2, and 15,960:1,024 (16:1) for rabbit #3. After a single booster, rabbit #1 gave a ratio of 6,190:1,024 (6:1). Ratio averages for the three rabbits indicate that the pre-booster bactericidal titers were 29 times greater than the corresponding maximum agglutinin titers.

Bactericidal antibodies were present naturally in the normal sera of the three rabbits even though agglutinins could not be detected. Production of bactericidal antibodies occurred a little sooner than did production of agglutinins although both antibodies appeared at their maximum concentrations in 10-18 days. The maximum serum levels of agglutinins, however, were slower to decline.

Response Slope

Table 2 contains the response slopes from each test listed in order of bleeding. These values ranged from a low of 0.91 before immunization for rabbit #3 to a high of 3.6 after immunization also for rabbit #3.

In all three rabbits the greatest slope matched the greatest antibody titer and the flattest slope matched the lowest antibody titer. An increase in slope occurred immediately after immunization and continued to rise until the antibody titer reached its maximum value.

In this particular study, the younger rabbits #1 and #3 exhibited the greatest changes in slope values and the older rabbit #2 the least. It may be that the two younger rabbits whose pre-immunization antibody titers were only 2 and 3 contained the fewest normal antibodies and therefore exhibited the greatest change in total serum action after immunization. The final antibody titer of rabbit #2 was more than twice that of rabbits #1 and #3 and yet it still contained the least increase in slope even though the pre-immunization antibody titer was only 1:10.

CHAPTER V

DISCUSSION OF TEST VARIABLES

Inoculum

Preliminary tests were conducted to determine whether or not the photometric assay test could be applied to the Salmonella pullorum system. Trypticase soy broth, a highly recommended media for culturing Salmonella antigen, was selected for growing the seed culture inoculum. Even though maximum bacterial growth was attained, the broth medium almost completely inhibited the killing action of the antibody plus complement. Mackie (1931 and 1932) had observed a similar inhibitory effect and attributed the cause to an extracellular product of bacterial growth which could be removed by washing the bacteria with physiological saline. Later investigations (Michael and Braun, 1959) demonstrated similar inhibitory effects of certain amino acids. Consequently, it was felt here that the peptones, trypticase and phytone present in the trypticase soy broth produced the undesirable effect.

Washing and reconstituting the overnight trypticase soy broth culture of Salmonella pullorum with physiological saline instead of broth removed most of this inhibition. However, serum titers were not as reproducible as they should have been and a lag of bacterial growth occurred in all of the tubes including the controls. This meant that an

additional hour or two was required to reach the same optical density obtained using identical concentrations of inoculum in broth.

Brain heart infusion broth was then substituted for trypticase soy broth and it was discovered that no inhibition of the bactericidal action took place. Brain heart infusion broth was, therefore, used throughout this study and the washing and diluting of bacteria with physiological saline omitted. The small amount of broth then present in the inoculum in each tube helped maintain the bacteria in a metabolically active state during the reaction time and prevented a lag of growth during the growth time. If no bacterial nutrients were present during the reaction time, the bacterial growth was retarded in all of the tests and controls.

The indicated antibody titer was an inverse function of the number of viable bacteria in the inoculum. For this reason it was of greatest importance to pipette exactly the same amount of inoculum into each tube. The standard culture of 72% transmittance was selected in order that growth would be rapid enough to permit final readings in a few hours. Plate counts have determined this concentration to contain about 1.75 X 10^8 viable bacteria per ml.

Absorption of Complement

Practically all normal guinea pig serum used as a complement source contained small concentrations of non-specific or specific antibodies against Salmonella pullorum. These antibodies could be removed

by absorption. Efficiency of the absorption process was measured by determining the killing power of the complement without any other source of antibody. A standard requirement for the absorbed complement reagent was set in this study so that 75% or more of the inoculated bacteria survived exposure to the complement as compared to a diluent control tube not containing complement. This standard was very difficult to consistently meet throughout this study. Using different lots of lyophilized guinea pig complement, survivals of 50% to 95% were attained at different times even though each process concisted of a double absorption. Some factors in technique which influenced, at least in part, the variations in removal of antibody were:

1. A virulent culture of Salmonella pullorum freshly isolated from a mouse passage was more active antigenically and far more effective in absorbing the antibodies than was an old stock culture maintained on routine laboratory media. During this study efficiency in absorption was restored by passing the stock culture through one or more mouse passages periodically.
2. Washing the Salmonella pullorum antigen with magnesium saline diluent rendered the bacteria more efficient in absorbing than did washing with distilled water.
3. Frequent transfer of Salmonella pullorum on blood agar plates helped to maintain the mouse passage bacteria in a more active state antigenically than did infrequent transfers.

Although careful following of the above mentioned conditions greatly aided in the efficiency absorption, the problem was not solved. Different lots and brands of complement varied significantly in antibody concentration and in retention of complement activity after the absorption process. One brand of complement, for example, was completely inadequate for use due to loss in complement activity while similar lots of Hyland and Difco complement were satisfactory. Also, retention of complement activity during storage of frozen absorbed complement varied significantly. One source of complement withstood appreciable loss in complement activity after storing frozen for three weeks while another brand was much less efficient after storing frozen for only two weeks.

Since a considerable portion of the complement prepared for use during this study did not meet the survival requirement of 75% or more bacteria, an attempt was made to determine whether or not the substandard complement could be used for antibody determinations. Paired antibody determinations from a single test serum were run using standard and substandard complement. It was found that the resulting antibody titers showed no significant differences and that the substandard complement could, therefore, be used for accurate serum antibody content determinations. The growth time incubation periods using substandard complement often needed to be extended for one or two hours,

however, in order to attain the same amount of growth in the complement controls as was attained using standard complement at the four and five hour intervals.

A third absorption would bring the substandard complement within the standard requirement range. However, each absorption process offered a little more opportunity for deterioration of complement activity and it was decided that in view of this fact and in view of the additional time and testing required, that a third absorption would be omitted.

Since bovine sera has been frequently recommended as a complement source for the bactericidal system, serum from a young heiffer was tested as a possible source of complement. However, absorption of the bovine serum was far less effective than absorption of guinea pig serum. Use of guinea pig was, therefore, continued despite the greater convenience in obtaining a bovine source.

Physiological State of Bacteria

Only bacteria in a physiologically active state of metabolism were susceptible to the killing effect of the antibody-complement system. It has been demonstrated that week old broth cultures are insusceptible to the killing action by antibody plus complement (Muschel, 1956). For this study bacteria used in the bactericidal test were kept in an active state of metabolism and great care was taken to make sure the inoculum

contained bacteria in the logarithmic growth phase. It was noted during this study that broth cultures inoculated with bacteria from old blood agar plates (several weeks) were less susceptible to killing by antibody plus complement than were fresher cultures.

CHAPTER VI

CONCLUSION OF RESULTS

This study was undertaken to demonstrate whether Salmonella pullorum was an organism susceptible to the killing action of antibody plus complement, and the value of this reaction as a sensitive test for specific antibody. Results indicate:

1. Salmonella pullorum was highly susceptible to the killing action of complement in the presence of specific antibody. This antibody was produced in high concentration in response to animal immunization.

2. Less antibody was required to sensitize Salmonella pullorum to the killing action of complement than was required to produce agglutination. Results of the rabbit immunization program indicated that the maximum bactericidal titers of rabbits were 69, 6, and 16 times greater than the corresponding agglutinin titers.

3. Bactericidal antibody appeared in the serum of the immunized animals slightly sooner and decreased much more rapidly than did the agglutinin antibody.

4. Salmonella pullorum antibody was present naturally in concentrations of 1:10 or less in all non-immunized rabbit, bovine, and guinea pig sera tested. Agglutinins were not present in detectable amounts.

5. A "prozone" or Neisser-Wechsberg effect occurred with hyperimmune rabbit sera such that larger dilutions of serum produced greater killing than did lesser dilutions. Undiluted serum usually produced no killing effect at all.

6. The age of the animal appeared to be a significant factor influencing production of antibodies in response to immunization in that the 8 month old rabbit produced a much higher bactericidal and agglutinin titer than did the two 6 week old rabbits.

7. The dosage of antigen appeared to influence the production of bactericidal antibodies sooner than agglutinins. The fourth largest dose of antigen appeared to absorb or cause a decline in bactericidal antibody titer while stimulating a further production of agglutinins.

8. Efficiency in absorbing specific antibody from guinea pig complement was greatly enhanced by using a smooth culture, freshly passed through a mouse, and by washing in mg-saline diluent rather than in saline or plain distilled water.

9. Bovine serum as a source of complement proved unsatisfactory because of the extreme difficulty in absorbing out specific antibody.

10. The physiological state of the bacterial seed culture greatly influenced the growth time. Seed inoculum washed and diluted

with physiological saline instead of brain heart infusion broth required a longer time for the control to reach the end point density concentration. Old cultures were not susceptible to the killing action of complement plus antibody.

11. Trypticase soy broth was unsatisfactory as media for growing S. pullorum antigen as the antibody complement reaction was inhibited. Brain heart infusion broth cultures did not contain significant inhibitory agents.

BIBLIOGRAPHY

Amano, T., Fujikawa, K., Morioka, T., Miyama, A., and Ichikawa, S. 1958. Quantitative studies on immune bacteriolysis. I. A new method of quantitative estimation. Bikens J. 1:13.

Bordet, Jules. 1895. Les Leucocytes et les propietes actives du serum chez les vaccines. Ann. Inst. Pasteur 9:462.

Boyd, William Clouser. 1956. Fundamentals of immunology. 3rd ed., 776 p. New York: Interscience Publishers.

Browning, C. H. and Mackie, T. J. 1949. Textbook of bacteriology. 11th ed. of Muir and Ritchie's manual, 907 p. London: Oxford University Press.

Fife, Earl H., Jr. and Muschel, Louis H. 1961. Influence of magnesium and calcium ions on the hemolytic activity and stability of guinea pig complement. J. of Immunol. 87:688.

Gordon, J. and Carter, H. A. 1932. The bactericidal power of normal serum. J. Path. Bact. 35:549.

Hook, W. and Muschel, L. 1959. Lysozyme and properdin levels in mice treated with zymosan, endotoxin or X-irradiation. Fed. Proc. 18:573.

Landy, Maurice, Michael, J., and Whitby, James L. 1962. Bactericidal method for the measurement in normal serum of antibody to gram-negative bacteria. J. Bact. 83:631.

Mackie, T. J. and Finkelstein, M. H. 1931. Natural bactericidal antibodies: Observations on the bactericidal mechanism of normal serum. J. Hyg. 31:35.

Mackie, T. J. and Finkelstein, M. H. 1932. The bactericidins of normal serum: Their characters, occurrence in various animals and the susceptibility of different bacteria to their action. J. Hyg. 32:1.

Mackie, T. J. and McCartney, J. E. 1953. Handbook of practical bacteriology. 9th ed., 677 p. Baltimore: Williams and Wilkins.

Metchnikoff, Elie. 1907. Immunity in infective diseases. 2nd ed., 591 p. Cambridge, England: Cambridge University Press.

Michael, Jacob G. and Braun, Werner. 1959. Modification of bactericidal effects of human sera. Proc. Soc. Exper. Biol. Med. 102:486.

Michael, Jacob G., Whitby, James L., and Landy, Maurice. 1962. Studies on natural antibodies to gram-negative bacteria. J. Exper. Med. 115:131.

Miles, A. A. and Misra, S. S. 1938. The estimation of the bactericidal power of the blood. J. Hyg. 38:732.

Muir, Robert and Browning, Carl Hamilton. 1908. On the bactericidal action of normal serum. J. Path. and Bact. 13:76.

Muir, R. 1931. A system of bacteriology in relation to medicine. Volume 6, 538 p. London: His Majesty's Stationery Office.

Muschel, Louis H. and Treffers, Henry P. 1952. Serum bactericidal activity by turbidimetric growth assay method. Fed. Proc. 11:477.

Muschel, Louis H. and Treffers, Henry P. 1956. Quantitative studies on the bactericidal actions of serum and complement. I. A rapid photometric growth assay for bactericidal activity. J. Immunol. 76:1.

Muschel, Louis H. and Treffers, Henry P. 1956. Quantitative studies on the bactericidal actions of serum and complement. II. Some implications for the mechanism of the bactericidal reaction. J. Immunol. 76:11.

Muschel, Louis H. and Treffers, Henry P. 1956. Quantiative studies on the bactericidal actions of serum and complement. III. Observations on sera obtained after T. A. B. vaccination or during typhoid fever. J. Immunol. 76:20.

Muschel, Louis H. and Muto, T. 1956. Bactericidal reaction of mouse serum. Science 123:62.

Muschel, Louis H., Chamberlin, Richard H., and Osawa, Etsuko. 1958. Bactericidal activity of normal serum against bacterial cultures. I. Activity against Salmonella typhi strains. Proc. Soc. Exper. Biol. and Med. 97:376.

Muschel, Louis H., Carey, W. F., and Baron, L. S. 1959. Formation of bacterial protoplasts by serum components. J. Immunol. 82:38.

Muschel, Louis H. 1960. Serum bactericidal actions. Ann. New York Acad. Sc. 88:1265.

Muschel, Louis H. 1960. Bactericidal activity of normal serum against bacterial cultures. II. Activity against Escherichia coli strains. Proc. Soc. Exper. Biol. and Med. 103:632.

Nagington, J. 1956. The bactericidal action of typhoid antibody. Brit. J. Exptl. Path. 37:385.

Osawa, Etsuko and Muschel, Louis H. 1959. Formation of bacterial protoplasts by serum components. J. Immunol. 82:38.

Osawa, Etsuko. 1960. Influence of temperature on the sensitivity of Paracolobactrum ballerup to the bactericidal action of serum. Fed. Proc. 19:206.

Osawa, Etsuko and Muschel, Louis H. 1960. The bactericidal action of normal serum and the properdin system. J. Immunol. 84:203.

Roantree, Robert J. and Pappas, Nicholas C. 1960. The survival of enteric bacilli in the blood stream as related to their sensitivity to the bactericidal effect of serum. J. Clin. Invest. 39:82.

Smith and Conant. 1960. Zinsser microbiology. 12th ed., 1026 P. New York: Appleton-Century-Crofts, Inc.

Toussaint, A. J. and Muschel, Louis H. 1959. Neutralization of bacteriophage by normal serum. Nature 183:1825.

Treffers, Henry P. and Yaw, Katherina E. 1948. A turbidimetric growth assay method for the determination of the relative bactericidal activities of sera. Fed. Proc. 7:311.

Treffers, Henry P. and Muschel, Louis H. 1954. The combined actions of chloramphenicol and of bactericidal antibody plus complement on Salmonella typhosa. J. Exper. Med. 99:155.

Wardlaw, Alastair C and Pillemer, Louis. 1956. The properdin system and immunity. V. The bactericidal activity of the properdin system. J. Exper. Med. 103:553.

Wardlaw, Alastair C. 1962. The complement-dependent bacteriolytic activity of normal human serum. I. The effect of pH and ionic strength and the role of lysozyme. J. Exper. Med. 115:1231.

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EVALUATION OF THE PHOTOMETRIC ASSAY METHOD TO QUANTITATE THE BACTERICIDAL ACTION OF NORMAL AND IMMUNE RABBIT SERA ON SALMONELLA PULLORUM

by

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ABSTRACT

The bactericidal antibody test depends on the presence of sufficient complement, magnesium ions, and specific antibody to produce killing of certain gram negative bacilli. This study was undertaken to demonstrate whether or not Salmonella pullorum is an organism which is susceptible to this killing action.

Different amounts of test serum containing antibody, constant amounts of complement and magnesium saline diluent were placed together and held in a 37°C water bath for a one hour reaction time. During this interval, the antibody-complement system was given an opportunity to kill the susceptible bacteria. Then, a constant volume of nutrient broth was added to each tube to terminate the killing action and to provide nutrients for the surviving bacteria to multiply as they were incubated at 37°C for a growth time.

At intervals of four and five hours incubation at 37°C the density of growth in each test and control tube was measured using a photoelectric colorimeter. During this time the bacteria were in the logarithmic phase of growth and the optical density was directly proportional to the number of bacteria surviving the original inoculum. Using this information it was possible to measure the apparent degree of killing produced by the test serum during the reaction time and to

quantitatively determine its antibody titer by calculating that amount of serum producing a 50% survival end point.

Immunization of three rabbits was conducted in order to demonstrate the effectiveness of the bactericidal system using Salmonella pullorum. Results indicated that Salmonella pullorum was highly susceptible to the killing action of the antibody system.

Bactericidal assays and agglutination tests were performed on the rabbit sera at regular intervals during the immunization program and the antibody titers compared. Results indicated that the bactericidal antibody test was indeed a very sensitive test for Salmonella pullorum antibody detection with maximum bactericidal titers of 17,540, 51,720, 15,960 resulting from the sera of rabbits #1, #2, and #3 as compared to maximum agglutination titers of 256, 8,192, and 1,024. Thus, for rabbits #1, #2, and #3 the greatest bactericidal antibody titer of the serum was 69, 6, and 16 times greater than the corresponding top agglutinin titer.